Regulation by gastric acid of the processing of progastrin-derived peptides in rat antral mucosa

J. A. Macro, G. W. Bate, A. Varro*, C. Vaillant †, N. G. Seidah ‡, R. Dimaline and G. J. Dockray

Physiological Laboratory and †Department of Veterinary Preclinical Science, University of Liverpool, Crown Street, PO Box 147, Liverpool L69 3BX, UK and ‡Laboratory of Neuroendocrinology, Clinical Research Institute of Montreal, Montreal, Canada

1. Inhibition of gastric acid secretion by proton pump inhibitors like omeprazole increases the synthesis and secretion of the pyloric antral hormone gastrin. We report here how omeprazole influences the conversion of the gastrin precursor to its final products, and the abundance of mRNAs encoding proteins associated with progastrin processing in rat antral mucosa.

2. Progastrin processing was studied using a pulse–chase protocol in antral mucosa, incubated *in vitro*, from rats treated with omeprazole for up to 5 days. Labelled peptides were detected by on-line scintillation counting after immunoprecipitation and HPLC. The mRNAs encoding prohormone-processing enzymes were identified by Northern blot, polymerase chain reaction or ribonuclease protection assay, and their cellular origins identified by immunocytochemistry.

3. Cleavage of [^{3}H]- and [^{35}S]-labelled progastrins at Arg-94–95 or Arg-57–58, and amidation at Phe-92 were not influenced by pretreatment with omeprazole. In contrast, cleavage of G34 (the thirty-four amino acid amidated gastrin) at Lys-74–75 to give G17 (the seventeen amino acid amidated gastrin), and of G34–Gly to G17–Gly (G34 and G17 with COOH-terminal glycine), was increased 3-fold after treatment with omeprazole for either 1 or 5 days.

4. Approximately 20% of newly synthesized amidated and Gly-extended gastrins were secreted within 240 min of the labelling period in omeprazole-treated samples, but secretion of labelled gastrins from control tissue was undetectable over a comparable period.

5. The amidating enzyme, peptidylglycine α-amidating mono-oxygenase (PAM), the prohormone convertases PC1/3, PC2, PC5 and the PC2 chaperone 7B2 were localized to rat antral gastrin cells by immunocytochemistry. The relative abundance of mRNA species encoding 7B2, PC5 and PAM were unchanged after treatment with omeprazole for 5 days, whereas gastrin, PC1/3 and PC2 mRNAs are known to increase at this time.

6. The main consequence of increased cleavage at Lys-74–75 is the production of G17 and G17–Gly at the expense of G34 and G34–Gly, respectively. The latter have longer plasma half-lives, and so their increased cleavage may serve to limit the rise in plasma gastrin concentration after inhibition of acid secretion. Changes in the abundance of mRNAs encoding prohormone-processing enzymes cannot account for the rapidity of the changes in cleavage of progastrin at Lys residues after omeprazole.

The pyloric antral hormone gastrin occurs in multiple active forms that differ in biological properties (Dockray, Varro & Dimaline, 1996). The COOH-terminally amidated peptides of thirty-four and seventeen amino acid residues (G34 and G17) stimulate both gastric acid secretion and the growth of gastric mucosal cells, particularly the histamine-producing enterochromaffin-like (ECL) cells of the gastric corpus (Gregory & Tracy, 1964, 1972; Larsson *et al.* 1986). Recent studies suggest that gastrins extended at the COOH-terminus by glycine (Gly-gastrins) are also growth factors (Seva,

* To whom correspondence should be addressed.

Dickinson & Yamada, 1994; Singh, Owlia, Espeijo & Dai, 1995). In addition they may modulate the action of G34 and G17 on acid secretion, but they do not stimulate acid secretion on their own (Todisco, Takeuchi, Seva, Dickinson & Yamada, 1995; Higashide, Gomez, Greeley, Townsend & Thompson, 1996). The various forms of gastrin are generated during biosynthesis from a common precursor, progastrin (Varro, Henry, Vaillant & Dockray, 1994; Varro, Voronina & Dockray, 1995), which is itself also now recognized to be a putative growth factor (Wang *et al.* 1996).

The initial product of gastrin mRNA translation is the precursor preprogastrin, which is co-translationally converted to progastrin (Fig. 1). The latter is sequestered in secretory granules and there converted to the Gly-gastrins and then amidated gastrins (Varro *et al.* 1994, 1995). The results of pulse–chase labelling experiments in rat antral mucosa indicate that G34–Gly is the first major intermediate generated from progastrin. Approximately 50% of G34–Gly is converted to G34 through the action of the COOH-terminal amidating enzyme, peptidylglycine α-amidating mono-oxygenase (PAM), and the remainder is cleaved to yield G17–Gly (Fig. 1). In principle the latter could be converted by PAM to G17, but this appears not to be a major route to G17, which instead is largely generated by cleavage of G34 (Varro *et al.* 1995). The main enzymes involved in cleavage of progastrin and its derivatives are thought to be members of the prohormone convertase (PC) family. Three members of the family, PC1/3, PC2 and PC5, are expressed in antral mucosa (Macro, Dimaline & Dockray, 1996), but the particular representatives of the PC family in rat gastrin cells (G-cells) are not known, nor for that matter has the precise identity of PAM in G-cells been established.

Gastrin secretion is regulated by the luminal contents of the stomach. Protein and protein digestion products stimulate gastrin release, and intragastric acid inhibits it (Walsh, Richardson & Fordtran, 1975; Walsh, 1994). When acid secretion is blocked, for example by proton pump inhibitors such as omeprazole, there is increased gastrin secretion (Larsson *et al.* 1986; Walsh, 1994). With prolonged achlorhydria, gastrin synthesis is also upregulated, which serves to maintain the increased rate of gastrin release (Brand & Stone, 1988; Wu, Giraud, Mogard, Sunii & Walsh, 1990; Dockray, Hamer, Evans, Varro & Dimaline, 1991). Thus, 24 h after administration of omeprazole in the rat, there is increased translation of gastrin mRNA, and after 48 h there is an increase in the abundance of gastrin mRNA (Brand & Stone, 1988; Wu *et al.* 1990; Bate, Varro, Dimaline & Dockray, 1996). The stores of gastrin in antral mucosa are scarcely changed in omeprazole-treated rats, so that in the presence of increased synthesis and release the time for progression of gastrin through the G-cell must be reduced (Dockray *et al.* 1991; Varro, Yegen & Dockray, 1993). This raises the question of whether there might be upregulation of the rates of progastrin processing in omeprazole-treated rats in order to complete the normal sequence of prohormone-processing events prior to secretion, and if so whether regulation is attributable to changes in the expression of prohormone-processing enzymes. We have examined this issue by comparison of the rates of progastrin processing, and the abundance of processing-enzyme transcripts in control rats and in rats treated with omeprazole for up to 5 days. We report here increased cleavage of G34 and G34–Gly in rats treated with omeprazole for 24 h, but no change in the kinetics of other processing steps. We describe the prohormone-processing enzymes found in G-cells, and the effect of omeprazole treatment on the abundance of their respective mRNA species.

METHODS

Animals and treatments

Male Wistar rats (initial body weight approximately 250 g) were maintained on a 12 h light–dark cycle, with food and water *ad libitum*. Omeprazole (a gift from Astra Hassle, Molndal, Sweden)
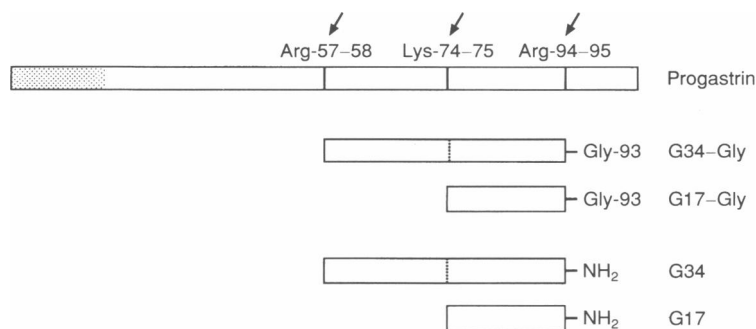

Figure 1. Schematic representation of the structural relationships between progastrin and its major products

Progastrin is generated from preprogastrin by removal of an NH_2-terminal signal peptide (stippled). Cleavage at pairs of arginine residues (Arg-57–58, Arg-94–95) by endo- and carboxypeptidase action generates G34–Gly. The latter may be either cleaved at a pair of lysine residues (Lys-74–75) to yield G17–Gly, or COOH-terminally amidated by peptidylglycine α-amidating mono-oxygenase (PAM) to generate G34, which in turn may be cleaved at lysines to yield G17.

was administered by gavage (400 μmol kg^{-1}, daily for up to 5 days); 24 h after the final treatment, animals were killed by cervical dislocation followed by decapitation for collection of trunk blood. Antral mucosa was dissected free of muscle for extraction of RNA or incubation *in vitro*.

Progastrin processing

Progastrin processing was examined using a pulse–chase experimental protocol as previously described (Varro *et al.* 1994, 1995). In brief, antral mucosal explants (equivalent to one rat antrum per sample) were incubated at 22 °C for 2 h in 3 ml modified Krebs–Ringer bicarbonate solution (composition (mM): 95 NaCl, 4·7 KCl, 2·6 CaCl$_2$, 1·2 KH$_2$PO$_4$, 24·9 NaHCO$_3$, 10 Hepes, 4·9 sodium pyruvate, 5·4 sodium fumarate, 4·9 sodium glutamate, 11·6 glucose) gassed with 95 % O$_2$–5 % CO$_2$ containing 50 μCi ml^{-1} [^{3}H]Tyr and 100 μCi ml^{-1} [^{35}S]sulphate (Amersham International plc); in these conditions newly synthesized, intact, progastrin accumulates in the *trans*-Golgi network (TGN) and more proximal compartments of the secretory pathway (Varro *et al.* 1994). Progastrin processing was initiated by raising the temperature to 37 °C and replacing the medium with one containing 0·1 mM tyrosine and 1·2 mM MgSO$_4$; in these conditions progastrin is rapidly translocated to secretory granules where further post-translational processing takes place (Varro *et al.* 1995; Dockray *et al.* 1996). Depending on the experiment, samples were taken after 20, 40, 80 and 160 min at 37 °C. Progastrin-derived peptides were subsequently extracted from tissue with boiling water and concentrated by adsorption to Sep-pak C18 cartridges (Waters Associates, Millipore Corp., Millford, MA, USA) as previously described (Varro *et al.* 1994). The eluates were serially immuno-precipitated with antisera binding (a) the COOH-terminus of progastrin, (b) the COOH-terminus of Gly-extended gastrins (i.e. G34–Gly and G17–Gly) and (c) the COOH-terminus of the amidated gastrins, i.e. G34 and G17 (Varro *et al.* 1995). Immuno-precipitates were solubilized by boiling in water and fractionated by reversed-phase HPLC with on-line scintillation counting using a gradient of acetonitrile in 0·05 M ammonium bicarbonate. Where appropriate, the data were corrected for spillover of 30 % of ^{35}S into the ^{3}H channel.

Extraction of RNA and Northern analysis

Total RNA was extracted from rat antral mucosa using guanidium isothiocyanate (4 M in 25 mM sodium acetate, pH 6·0, containing 0·84 % (v/v) β-mercaptoethanol). mRNA was extracted using a polyAT tract kit (Promega, Southampton, UK). For Northern analysis, we used probes generated from the following: a full-length clone encoding mouse PC5 (Lusson, Vieau, Hamelin, Day, Chretien & Seidah, 1993), a cloned fragment corresponding to the mono-oxygenase domain of PAM (see below), cloned fragments of PC1/3, PC2 and gastrin cDNA (Macro *et al.* 1996), and a full-length clone encoding 7B2 (a gift from Dr J. Dixon, University of Michigan, Ann Arbor, MI, USA) (Waldbieser, Aimi & Dixon, 1991). Routinely, total RNA (20 μg) was separated by electrophoresis in a 1·0 % agarose gel containing formaldehyde; in addition, for Northern analysis of PAM mRNA, we separated mRNA on a 3 % acrylamide gel. Samples were electroblotted onto a nylon membrane (Nytran 13; Schleicher and Schuell, Dassel, Germany). Membranes were hybridized with cRNA probes generated from cDNA clones (2 × 10^6 c.p.m. ml^{-1}) at 68 °C in 50 % formamide followed by washing in high stringency conditions at 68 °C. Washed membranes were exposed to Phosphor storage screens (Molecular Dynamics, Sevenoaks, UK) for up to 3 days and signals visualized with a PhosphorImager and quantified using ImageQuant software

(Molecular Dynamics). Membranes were stripped of labelled probe by boiling in 0·1 % SDS and were sequentially examined with probes for other PC mRNAs, gastrin mRNA or 18s ribosomal subunit as previously described (Dimaline, Evans, Varro & Dockray, 1991; Macro *et al.* 1996). The latter was used as a reference marker for quantification as appropriate.

Identification of PAM transcripts

Different PAM transcripts were characterized by cloning and sequencing of polymerase chain reaction (PCR) products, and by ribonuclease protection assays (RPA). Antral total RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase and oligo(dT)$_{15}$ primer (Promega). Amplification by PCR was performed using standard Taq buffer (Promega) with the addition of 10 % glycerol (30 cycles, denaturation 95 °C, 1 min; annealing 50 °C, 1 min; extension 72 °C, 2 min). The primers for PCR were designed from the published sequence for rat atrial PAM (Stoffers, Green & Eipper, 1989) and corresponded to regions spanning the following functional domains of PAM: the peptidylglycine α-hydroxylating mono-oxygenase (PHM) domain (nucleotides 482–500 and 1012–1030, primers 1 and 2, respectively), the protease-sensitive domain (nucleotides 1490–1508 and 1770–1788, primers 4 and 5, respectively) and the transmembrane domain (nucleotides 2760–2778 and 3173–3192, primers 6 and 7, respectively). In addition, different species of PAM were distinguished using primer 7 and a primer (3) to nucleotides 1454–1473. The products of PCR were ligated into the pCRII plasmid (Invitrogen BV, NV Leek, Netherlands) and clones were sequenced using the dideoxy-chain termination reaction (sequenase II). For RPA, we used probes derived from the cloned products of primers 3 and 5, and primers 6 and 7. Total RNA (20 μg) was hybridized overnight at 42 °C with [^{32}P]cRNA (10 fmol) in hybridization buffer (80 % formamide, 100 mM sodium citrate, 300 mM sodium acetate, 1 mM EDTA, pH 6·4), and unhybridized single-stranded RNA was subsequently digested with RNase A (2·5 units ml^{-1}) and RNase T1 (100 units ml^{-1}) at 30 °C for 20 min. Protected RNA fragments were separated by 6 % polyacrylamide gel electrophoresis. Gels were dried and exposed to Phosphor storage screens as previously described.

Immunocytochemistry

Samples of antral mucosa were fixed by immersion in 4 % para-formaldehyde in 0·1 M sodium cacodylate, pH 7·4, and processed for cryostat sectioning. Sections were incubated at 4 °C for 18 h in a mixture consisting of mouse monoclonal antibodies against the amidated COOH-terminus of gastrin (antibody 28.2; diluted 1 : 50 000; a gift from J. H. Walsh, UCLA, Los Angeles, CA, USA) and rabbit polyclonal antisera (diluted 1 : 200 to 1 : 1000) against either the prohormone convertases PC1/3, PC2 or PC5, the PC2 chaperone protein 7B2 (a gift from Dr G. Martens, University of Nijmegen, The Netherlands), or PAM (471; a gift from Dr B. Eipper, Johns Hopkins Medical Institute, Baltimore, MD, USA). Antibody binding was identified using goat anti-mouse IgG conjugated to Texas Red (Vector Labs, Peterborough, UK) and biotinylated goat anti-rabbit IgG followed by streptavidin conjugated to fluorescein isothiocyanate (Amersham).

Radioimmunoassay

Plasma gastrin was determined by radioimmunoassay using an antiserum specific for the COOH-terminus of G17, and ^{125}I-G17 label (Dockray *et al.* 1991). Standard curves were prepared in rat plasma stripped of gastrin and samples were diluted so that they inhibited binding of label to antibody in the mid-region of the standard curve.

RESULTS

Progastrin processing in antrum from control and omeprazole-treated rats

When antral mucosa from both control and omeprazole-treated rats was incubated at 22 °C, there was labelling of intact progastrin with [³H]Tyr and [³⁵S]sulphate as previously reported (Varro *et al.* 1995; Bate *et al.* 1996). Raising the temperature to 37 °C coincident with removal and replacement of the media was associated with rapid disappearance of labelled progastrin at rates that were similar in tissue from control and omeprazole-treated rats (half-life ($t_{1/2}$) of 8 min for ³⁵S-labelled progastrin in controls, and 9 min in treated rats; a $t_{1/2}$ of 18 min for ³H-labelled progastrin, in both cases) (Fig. 2). The difference in rates of cleavage of ³H- compared with ³⁵S-progastrin is attributable to the accumulation of ³H-progastrin in the more proximal parts of the Golgi complex compared with ³⁵S-progastrin, which is only generated in the *trans*-Golgi network (Varro *et al.* 1994).

The main biosynthetic intermediate generated by cleavage of progastrin is G34–Gly, which is a substrate for the amidating enzyme PAM (Varro *et al.* 1995). The time course of appearance of COOH-terminally amidated gastrins was similar in control and omeprazole-treated rats. In both cases, approximately 60 % of Gly-gastrins were converted to amidated gastrin and this conversion was completed within 80 min of the initiation of progastrin cleavage (Fig. 2).

Cleavage of G34 and G34–Gly at a pair of Lys residues (positions 74 and 75 in preprogastrin) yields G17 and G17–Gly, respectively. In contrast to the data outlined above, there were significant differences in cleavage at Lys-74–75 in antral mucosa from control and omeprazole-treated rats. Thus in control rats, approximately 40 % of G34 was cleaved to G17 within 80 min of starting the chase compared with 70 % in omeprazole-treated rats (Fig. 3). Similarly, the ratio of G17–Gly to G34–Gly was lower in control rats than in omeprazole-treated animals (Fig. 3). It is known that gastrin synthesis and release are increased within 24 h of administering omeprazole (Bate *et al.* 1996), and we therefore examined whether cleavage rates might be changed over this period. In rats treated with omeprazole for 24 h, the ratios of G17 to G34 and G17–Gly to G34–Gly were significantly increased compared with controls (Fig. 4).

Kinetics of secretion

Plasma gastrin in rats fed *ad libitum* was 51·3 ± 5·6 pM. In rats treated with omeprazole for 1 day the plasma gastrin level was 208·2 ± 18·1 pM and after omeprazole for 5 days it was 353·6 ± 36·9 pM. In order to determine whether there was more rapid secretion of newly synthesized progastrin-derived peptides in omeprazole-treated samples as predicted (Dockray *et al.* 1991), we examined the appearance of labelled gastrins in the media in a pulse–chase protocol. In control rats there was no detectable secretion of labelled amidated or Gly-extended gastrins up to 240 min after initiating a chase (< 5 % of cellular content). In contrast, we consistently found secretion of labelled amidated and Gly-gastrins in antral mucosa of rats treated with omeprazole for 5 days: after 240 min incubation 18·0 ± 2·2 % of labelled amidated gastrins in tissue was secreted, and 9·3 ± 1·7 % of Gly-gastrins (*P* < 0·05 compared with control).

Localization of prohormone convertases to the G-cell

The putative prohormone convertases in G-cells were identified by immunocytochemistry. Antibodies to PC1/3, PC2 and PC5, and the amidating enzyme PAM, all labelled
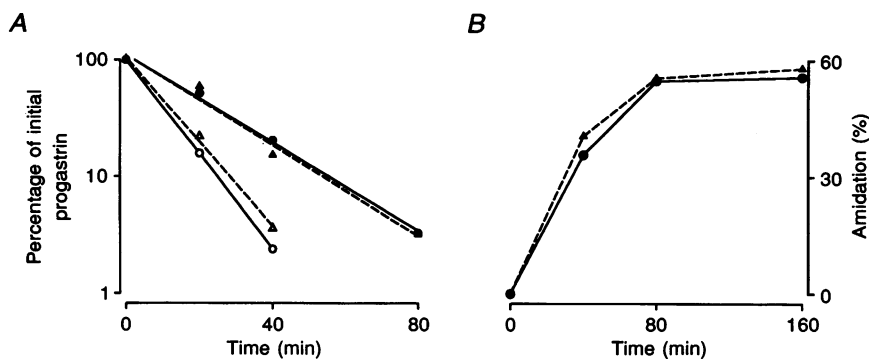

Figure 2. Effect of omeprazole on cleavage and amidation of progastrin-derived peptides

A, disappearance of [³H]tyrosine-labelled progastrin (filled symbols) and [³⁵S]sulphate-labelled progastrin (open symbols) in a pulse–chase experiment in antral mucosa from control rats (circles) and rats treated with omeprazole for 5 days (triangles). Isotope was incorporated into progastrin by incubation at 22 °C for 2 h, and then chased at 37 °C in fresh medium. The time from initiating the chase is indicated on the *x*-axis. The loss of labelled progastrin is represented on a log scale relative to the abundance of labelled progastrin at the end of the labelling period. Integrated counts are shown for immunoprecipitated progastrin after HPLC with on-line scintillation counting. *B*, appearance of ³H-labelled amidated gastrins timed from the start of the chase phase. Integrated data for ³H-labelled amidated gastrins (i.e. G17 and G34) as a percentage of total ³H-labelled amidated and Gly-extended gastrin. ●, control; ▲, rats treated for 5 days with omeprazole.
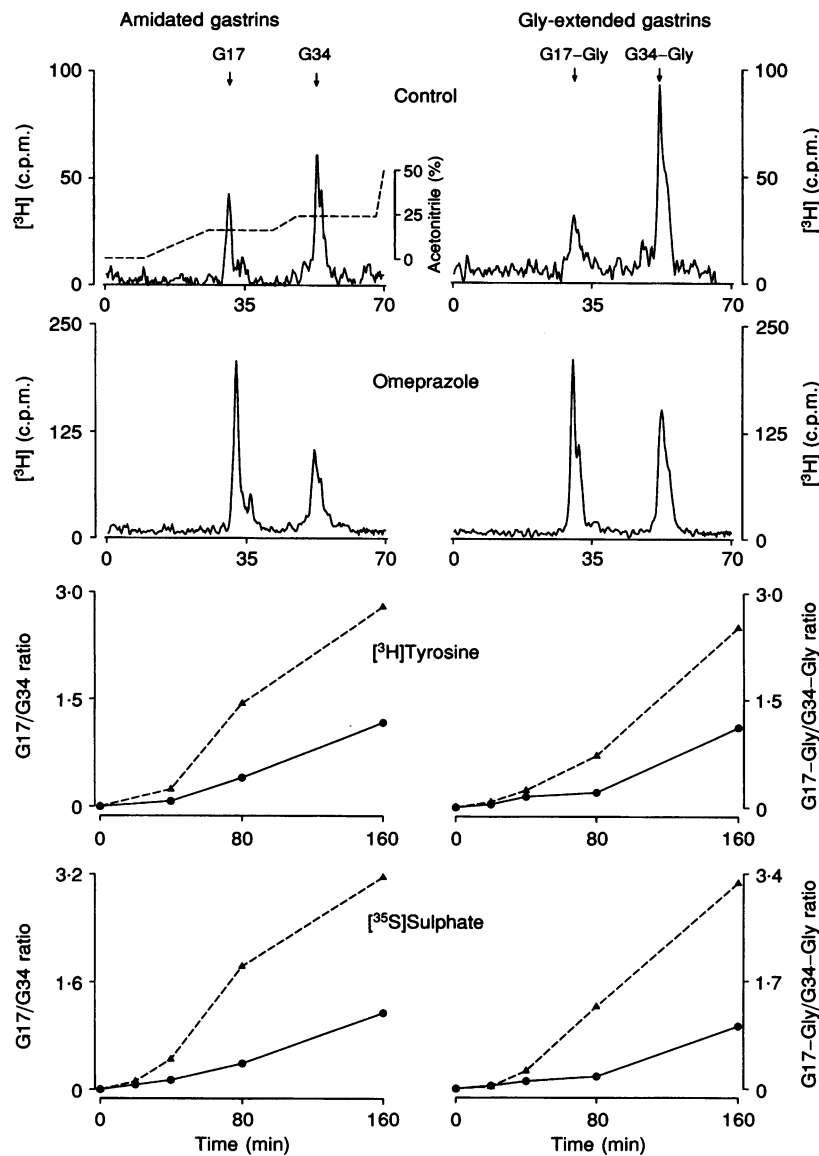
Figure 3. Cleavage of G34 and G34–Gly in control and omeprazole-treated rats

Representative HPLC traces are shown for ^{3}H-labelled amidated gastrins (left) and Gly-gastrins (right) from control rats (top) and rats treated with omeprazole (second panel down). Samples were taken 80 min after starting the chase. Arrows at the top indicate the identification of peaks. Dashed line in top left panel indicates acetonitrile gradient. In the bottom four panels, the integrated data from a study of the time course during the chase are shown for ^{3}H-labelled peptides and ^{35}S-labelled peptides. ●, control; ▲, rats treated for 5 days with omeprazole. Note that the ratio of G17 to G34, and of G17–Gly to G34–Gly is increased in omeprazole-treated rats.

Figure 4. Influence of treatment with omeprazole for 1 and 5 days on cleavage at Lys–Lys in progastrin-derived peptides

Cleavage at Lys-74–75 expressed as the ratio of G17 to G34 (A) and G17–Gly to G34–Gly (B), for ^{3}H-labelled peptides after 80 min chase in control rats (Con) and rats treated for 1 or 5 days with omeprazole. Results are expressed as means + S.E.M., $n = 6$. * $P < 0.05$, ANOVA.
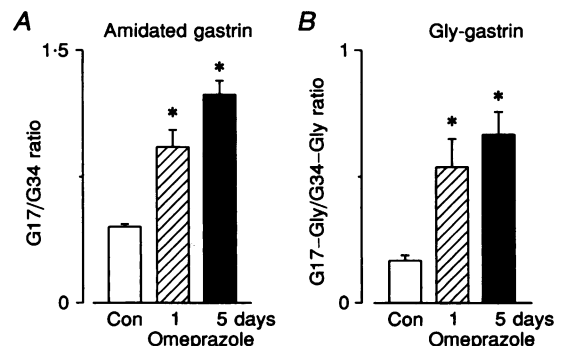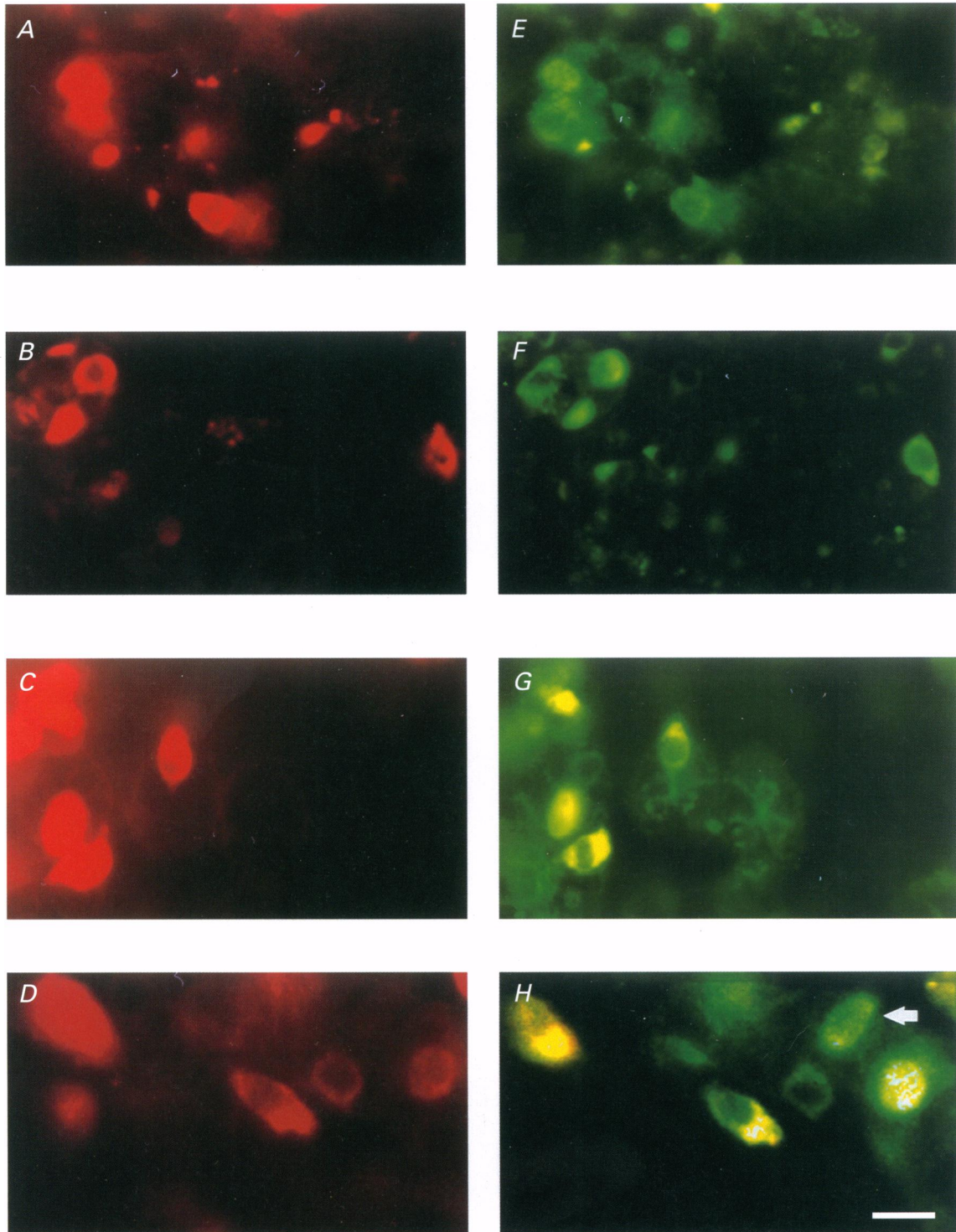
Figure 5. Immunocytochemical localization of processing enzymes in G-cells

Double immunofluorescent staining for gastrin and prohormone-processing enzymes in rat antral mucosa. *A–D*, G-cells identified by gastrin antibodies; *E–H*, G-cells in the same sections identified by antibodies to PC2 (*E*), PAM (*F*), PC5 (*G*) and PC1/3 (*H*). A small population of cells had PC1/3 immunoreactivity but not gastrin immunoreactivity (arrow in *H*). Scale bar, 12·5 μm.

G-cells (Fig. 5). The PC2 chaperone protein, 7B2, is already known to occur in antral endocrine cells (Falgueyret, Marcinkiewicz, Benjannet, Cantin, Seidah & Chretien, 1987) and we confirmed its presence in G-cells (not shown). The pattern of labelling was similar in control and omeprazole-treated rats.

Effects of omeprazole on prohormone convertase mRNA abundance

In rats treated with omeprazole for 5 days there was a modest increase in the abundance of mRNA encoding PC5 ($33 \pm 17\%$ compared with control), but there was no change after 1 day (Fig. 6). The mRNA encoding the PC2 chaperone protein, 7B2, did not change with omeprazole treatment although as previously noted there were significant increases in PC1/3 and PC2 after 5 days omeprazole, and as expected, gastrin mRNA abundance was increased about 3-fold in these samples (Macro *et al.* 1996).

PAM transcripts

In order to understand how gastrin amidation might be determined in rat antral mucosa we sought to characterize the mRNA species encoding PAM. In PCR using primers spanning the four functional domains of PAM (lyase, oxygenase, membrane-spanning and protease-sensitive domains) we found products corresponding to each domain (Fig. 7). Primers spanning the transmembrane domain yielded multiple PCR products that on the basis of size and sequence were identified as originating from PAM1, PAM2,

PAM3 or PAM3b (Stoffers, Ouafik & Eipper, 1991). In Northern analysis of mRNA using a probe to the mono-oxygenase domain we were able to separate two bands. Ribonuclease protection assays using a riboprobe corresponding to the protease-sensitive domain, which is present only in PAM1, indicated a band of the predicted size. In addition, RPA using a probe corresponding to the transmembrane domain of PAM1/2 gave rise to several bands. One of these (433 base pairs (bp)) could be generated from either PAM1 or PAM2. A second fragment (235 bp) would be generated only from PAM3b and a co-product in this case would be 144 bp, which was also identified. The latter could, however, be generated from PAM3 as well. We could not identify a product corresponding to PAM3a, i.e. 196 bp (Fig. 8). Together the data unequivocally support the presence in rat antrum of major transcripts corresponding to PAM1 and PAM3b; the presence of PAM2 and PAM3 is indicated by the PCR observations and is not excluded by the RPA data, while there is no evidence for the presence of PAM3a. The relative abundance of PAM transcripts detected in Northern blots did not change in rats treated with omeprazole for 5 days compared with controls (Fig. 6).

DISCUSSION

The present studies indicate that after inhibition of gastric acid secretion there is increased cleavage at Lys-74–75 in progastrin-derived peptides in rat pyloric antral G-cells. This response is selective since there is no evidence of
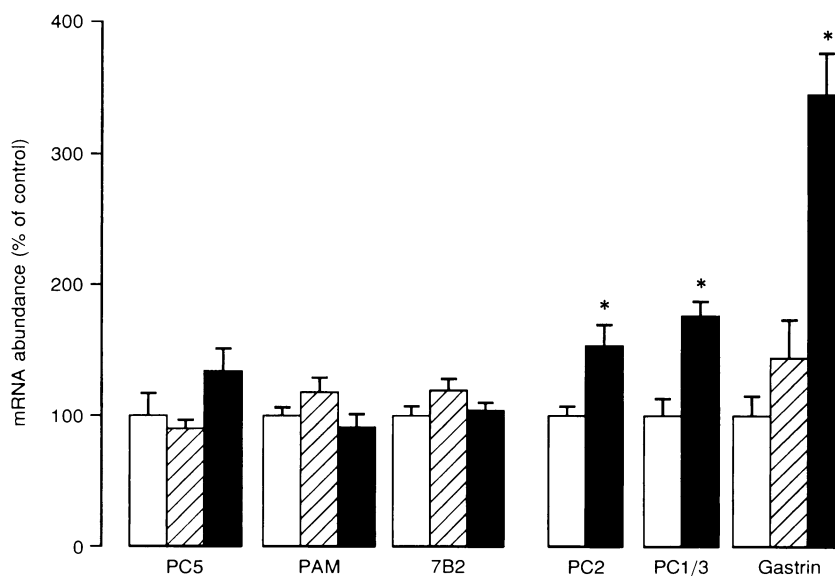

Figure 6. Relative abundance of mRNA species encoding prohormone convertases in antral mucosa of control and omeprazole-treated rats

The abundance of each mRNA species in antral extracts of rats treated with omeprazole for 1 or 5 days is expressed relative to that in control rats fed *ad libitum* (100%). Values are means + S.E.M., $n = 6$; $*P < 0.05$ compared with control, ANOVA). □, rats fed *ad libitum*; ▨, rats treated for 1 day with omeprazole; and ■, rats treated for 5 days with omeprazole.

changes in the rate of cleavage of progastrin at Arg-94–95, or of COOH-terminal amidation, after 5 days of omeprazole treatment. The cleavage and amidation of progastrin-derived peptides is known to occur in secretory granules (Dockray *et al.* 1996). However, the stores of gastrin in these granules are scarcely changed by achlorhydria, and consequently the time that gastrin is resident in secretory granules is greatly reduced in this condition (Dockray *et al.* 1991). Cleavage of Lys-74–75 is the slowest of the progastrin cleavages (Varro *et al.* 1995), and its increase with achlorhydria could provide a mechanism to allow completion of the full repertoire of prohormone processing events prior to secretion.

The inhibition of G-cell function by intragastric acid has been recognized for many years (Walsh *et al.* 1975; Walsh, 1994). Prolonged suppression of acid secretion is a strong stimulant of gastrin synthesis and release. In the rat, plasma concentrations rise 8- to 10-fold after 5 days of achlorhydria. Tissue gastrin stores are adequate to maintain normal plasma gastrin concentrations for about 20 h (Dockray *et al.* 1991) and in the achlorhydric stomach there are therefore increases in gastrin synthesis to maintain plasma gastrin concentrations. We and others have shown that gastrin mRNA abundance is increased in omeprazole-treated rats (Brand & Stone, 1988; Wu *et al.* 1990; Dimaline *et al.* 1991); however, the changes are slower than those in plasma gastrin and reach about 3-fold after 5 days. In contrast, within 24 h there is a 2-fold increase in gastrin mRNA translation rates, and after 5 days there is a 6-fold increase in mRNA translation (Bate *et al.* 1996). Evidently, therefore, the G-cell response to achlorhydria includes rapid modulation of gastrin release, followed by increased gastrin mRNA translation and, after about 2 days, increased gene
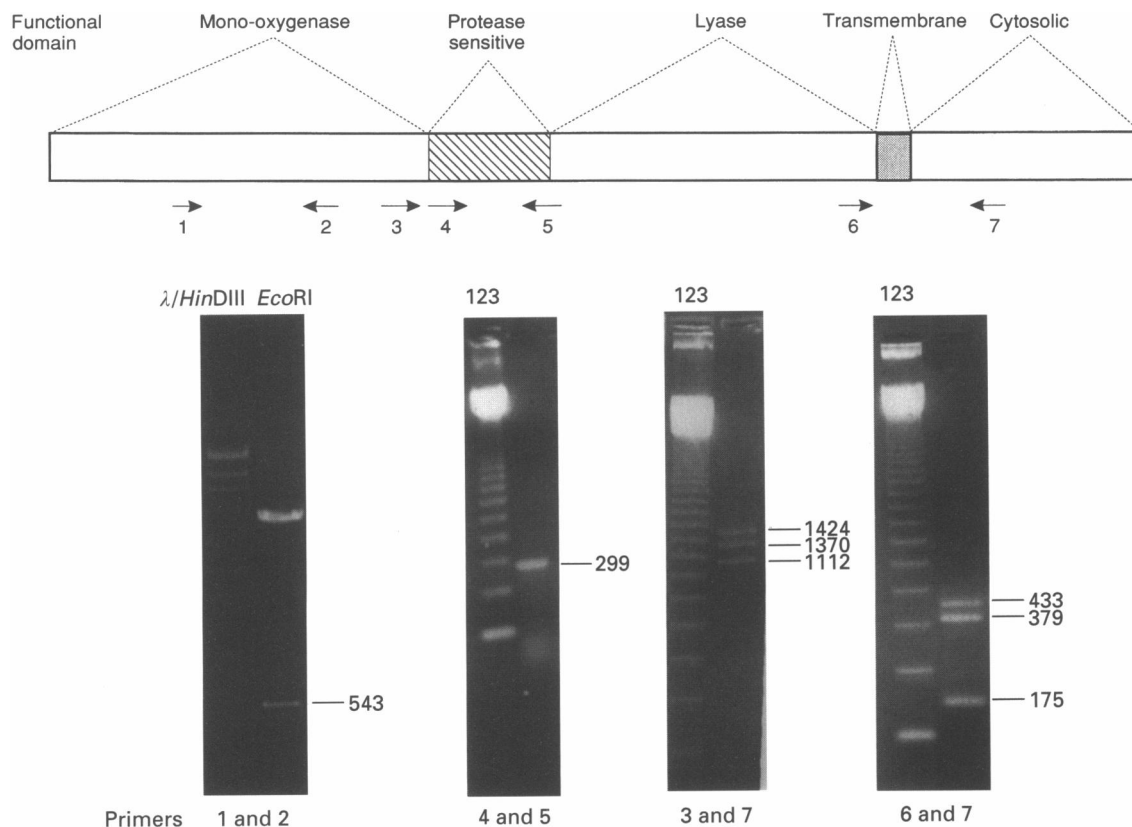

Figure 7. PCR identification of PAM transcripts

A schematic representation of the functional domains of PAM mRNA is shown at the top, together with arrows indicating the primers used for PCR. The results of PCR are shown at the bottom. In each case, a lane containing size markers (left) is shown together with one showing separation of the PCR products (right); the size of the observed products is indicated on the right. Primers 1 and 2 yielded a product corresponding to the expected size of the mono-oxygenase domain; the original PCR products were in low abundance and the figure shows cloned products recovered in restriction digests (note the identity of the cloned product was verified by sequencing). Primers 4 and 5 produced the expected product from the protease-sensitive region; primers 3 and 7, which span the lyase domain, yielded three bands the sizes of which correspond to the predicted products of PAM2 (1424 bp), PAM3b (1370 bp) and PAM3 (1112 bp). The latter primers did not yield products corresponding to PAM1 or PAM3a (predicted sizes, 1739 and 1166 bp, respectively). Primers 6 and 7 yielded three bands corresponding to the transmembrane domains of PAM1 or PAM2 (433 bp), PAM3b (379 bp) and PAM3 (175 bp); these primers did not yield a product characteristic of PAM3a (237 bp).

transcription. The results of the present study indicate that there is also control of selected steps in the pathway by which progastrin is processed to its active products. The amidated gastrins G34 and G17 have equal affinity for gastrin-CCK$_B$ receptors, but G34 is cleared approximately 5 times more slowly than G17 (Walsh, Debas & Grossman, 1974; Walsh, Isenberg, Ansfield & Maxwell, 1976; Eysselein, Maxwell, Reedy, Wunsch & Walsh, 1984). Similar rates of secretion of G34 and G17 therefore lead to 5 times higher plasma concentrations of G34. The increased rate of Lys–Lys cleavage in omeprazole-treated rats may serve to limit the absolute rise in plasma concentrations by ensuring that the major secreted product is G17.

Several putative prohormone convertases have now been characterized (Smeekens, Avruch, LaMendola, Chan & Steiner, 1991; Seidah *et al.* 1991, 1996). In the present study we showed that three PCs, PC1/3, PC2 and PC5, are present in G-cells. The maturation and activity of PC2 is thought to depend on a chaperone protein known as 7B2 (Braks & Martens, 1994; Martens, Braks, Eib, Zhou & Lindberg, 1994; Benjannet, Savaria, Chretien & Seidah,
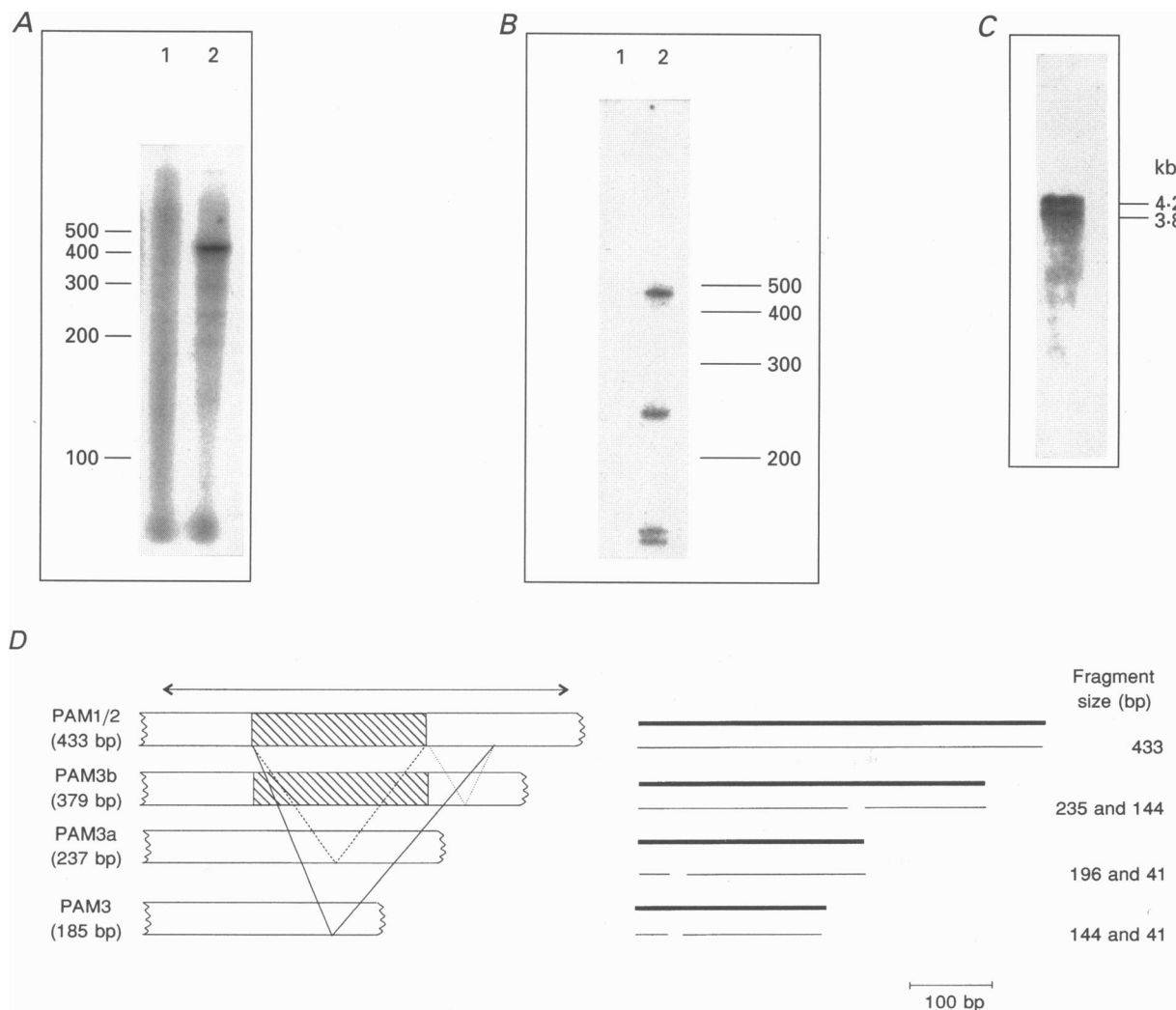

Figure 8. Assay of PAM transcripts

A, ribonuclease protection assay of PAM transcripts in rat antral mucosa, hybridized with a riboprobe corresponding to the protease-sensitive domain of PAM1 (lane 2); in *A* and *B* lane 1 shows yeast total RNA and lane 2 a ribonuclease-protected product of the size predicted. *B*, ribonuclease protection assay of PAM transcripts in rat antral mucosa using a riboprobe corresponding to the transmembrane domain of PAM1/2; note multiple bands (see *D* for a schematic representation of the predicted size of each band) compatible with the presence of either PAM1 or PAM2 (433 bp), PAM3b (235 bp) and either PAM3b or PAM3 (144 bp); there is no product corresponding to PAM3a (196 bp). *C*, Northern blot of PAM mRNA in rat antral mucosa showing that this approach resolves two bands. *D*, schematic representation of, left, the transmembrane domains of PAM1/2 and PAM3b (hatched bar) identifying the regions removed by mRNA splicing; on the right, the thick bars indicate the corresponding mRNA chain lengths and the thin bars indicate the predicted labelled products after ribonuclease protection. The predicted sizes are shown.

1995; Zhu & Lindberg, 1995), and this too has been localized to G-cells. Conceivably, changes in the expression of any of these molecules could modulate progastrin processing. We have previously reported that in rats treated with omeprazole for 5 days, there is upregulation in antral mucosa of the abundance of mRNA encoding two prohormone convertases, PC1/3 and PC2 (Macro *et al.* 1996). The present data indicate that mRNAs encoding other proteins that might regulate progastrin cleavage, such as PC5 and 7B2, are not appreciably changed after 5 days of treatment with omeprazole. Moreover, there is no evidence for any change in prohormone convertase mRNA abundance after 24 h omeprazole (Macro *et al.* 1996), when there is already increased Lys–Lys cleavage. The data indicate, therefore, that modulation of PC gene expression is unlikely to be responsible for the relatively rapid changes in gastrin processing with omeprazole treatment. In this context it is worth noting that in other systems, e.g. pituitary and endocrine pancreas, there is also regulation of PC mRNA abundance. Modulation of transcription may be important for long-term control of PC synthesis, but short-term responses to cell stimulation in both pancreas and pituitary appear to be attributable to increased translation of mRNA rather than increased transcription (Day, Schafer, Watson, Chretien & Seidah, 1992; Alarcon, Lincoln & Rhodes, 1993; Schuppin & Rhodes, 1996; Oyarce, Hand, Mains & Eipper, 1996).

Amidation at the COOH-terminus of gastrin determines which of two alternative types of biological activity is generated. The amidated gastrins are acid secretagogues and growth factors; their immediate precursors, the Gly-gastrins, are growth factors but do not stimulate acid secretion (Walsh, 1994; Seva *et al.* 1994). There may be interactions between the two types of progastrin product (Todisco *et al.* 1995; Higashide *et al.* 1996). We found that although the total synthesis of gastrin is increased with omeprazole treatment, the ratio of Gly-gastrin to amidated gastrin is maintained. In both control and omeprazole-treated rats, amidation occurs only in early or immature secretory granules. It is not clear what limits amidation to the period of approximately 80 min after formation of the granule. Our data suggest that both catalytic domains of PAM are expressed in antral mucosa and that both membrane-bound and soluble forms of the enzyme are present. Moreover, the maintenance of the amidation reaction in the face of increased flux of progastrin-derived peptides along the secretory pathway is evidently not due to changes in PAM mRNA type or abundance. It seems likely that the limitation to gastrin amidation that occurs in rat G-cells is due to some other factor. Addition of co-factors such as ascorbate do not change the kinetics of amidation in the present system (authors' unpublished observations) and further work is needed to identify the regulatory factors.

In conclusion, the present data indicate that acid inhibition of G-cell function involves the control of release of gastrin, of mRNA abundance and of selected post-translational modifications of progastrin. We suggest that the luminal environment of the stomach is therefore able to influence the forms of gastrin secreted by the G-cell and that this is part of the adaptive response to achlorhydria. The changes in progastrin processing in response to achlorhydria occur more rapidly than can be accounted for by changes in the expression of the processing enzymes; further work is now needed to determine the relevant mechanisms.

ALARCON, C., LINCOLN, B. & RHODES, C. J. (1993). The biosynthesis of the subtilisin-related proprotein convertase PC3, but not that of the PC2 convertase, is regulated by glucose in parallel to proinsulin biosynthesis in rat pancreatic islets. *Journal of Biological Chemistry* **268**, 4276–4280.

BATE, G. W., VARRO, A., DIMALINE, R. & DOCKRAY, G. J. (1996). Control of preprogastrin mRNA translation by gastric acid in the rat. *Gastroenterology* **111**, 1224–1229.

BENJANNET, S., SAVARIA, D., CHRETIEN, M. & SEIDAH, N. G. (1995). 7B2 is a specific intracellular binding-protein of the prohormone convertase PC2. *Journal of Neurochemistry* **64**, 2303–2311.

BRAKS, J. A. M. & MARTENS, G. J. M. (1994). 7B2 is a neuroendocrine chaperone that transiently interacts with prohormone convertase PC2 in the secretory pathway. *Cell* **78**, 263–273.

BRAND, S. J. & STONE, D. L. (1988). Reciprocal regulation of antral gastrin and somatostatin gene expression by omeprazole-induced achlorhydria. *Journal of Clinical Investigation* **82**, 1059–1066.

DAY, R., SCHAFER, M. K. H., WATSON, S. J., CHRETIEN, M. & SEIDAH, N. G. (1992). Distribution and regulation of the prohormone convertases PC1 and PC2 in the rat pituitary. *Molecular Endocrinology* **6**, 485–497.

DIMALINE, R., EVANS, D., VARRO, A. & DOCKRAY, G. J. (1991). Reversal by omeprazole of the depression of gastrin cell function by fasting in the rat. *Journal of Physiology* **433**, 483–493.

DOCKRAY, G. J., HAMER, C., EVANS, D., VARRO, A. & DIMALINE, R. (1991). The secretory kinetics of the G cell in omeprazole-treated rats. *Gastroenterology* **100**, 1187–1194.

DOCKRAY, G. J., VARRO, A. & DIMALINE, R. (1996). Gastric endocrine cells: gene expression, processing and targeting of active products. *Physiological Reviews* **76**, 767–798.

EYSSELEIN, V. E., MAXWELL, V., REEDY, T. J., WUNSCH, E. & WALSH, J. H. (1984). Similar acid stimulatory potencies of synthetic human big and little gastrins in man. *Journal of Clinical Investigation* **73**, 1284–1290.

FALGUEYRET, J. P., MARCINKIEWICZ, M., BENJANNET, S., CANTIN, M., SEIDAH, N. G. & CHRETIEN, M. (1987). Immunocytochemical localisation of a novel pituitary polypeptide 7B2 in the gastrointestinal tract of the rat. *Cell and Tissue Research* **249**, 707–709.

GREGORY, R. A. & TRACY, H. J. (1964). The constitution and properties of two gastrins extracted from hog antral mucosa. *Gut* **5**, 103–114.

GREGORY, R. A. & TRACY, H. J. (1972). Isolation of two 'big gastrins' from Zollinger-Ellison tumour tissue. *Lancet* **ii**, 797–799.

HIGASHIDE, S., GOMEZ, G., GREELEY, G. H., TOWNSEND, C. M. & THOMPSON, J. C. (1996). Glycine-extended gastrin potentiates gastrin-stimulated gastric acid secretion in rats. *American Journal of Physiology* 270, G220–224.

LARSSON, H., CARLSSON, E., MATTSSON, H., LUNDELL, L., SUNDLER, F., SUNDELL, G., WALLMARK, B., WATANABE, T. & HAKANSON, R. (1986). Plasma gastrin and gastric enterochromaffinlike cell activation and proliferation: studies with omeprazole and randitidine in intact and antrectomized rats. *Gastroenterology* 90, 391–399.

LUSSON, J., VIEAU, D., HAMELIN, J., DAY, R., CHRETIEN, M. & SEIDAH, N. G. (1993). cDNA structure of the mouse and rat subtilisin/kexin-like PC5: A candidate proprotein convertase expressed in endocrine and nonendocrine cells. *Proceedings of the National Academy of Sciences of the USA* 90, 6691–6695.

MACRO, J. A., DIMALINE, R. & DOCKRAY, G. J. (1996). Identification and expression of prohormone-converting enzymes in the rat stomach. *American Journal of Physiology* 279, G87–93.

MARTENS, G. J. M., BRAKS, J. A. M., EIB, D. W., ZHOU, Y. & LINDBERG, I. (1994). The neuroendocrine polypeptide 7B2 is an endogenous inhibitor of prohormone convertase PC2. *Proceedings of the National Academy of Sciences of the USA* 91, 5784–5787.

OYARCE, A. M., HAND, T. A., MAINS, R. E. & EIPPER, B. A. (1996). Dopaminergic regulation of secretory granule-associated protein in rat intermediate pituitary. *Journal of Neurochemistry* 67, 229–241.

SCHUPPIN, G. T. & RHODES, C. J. (1996). Specific coordinated regulation of PC3 and PC2 gene-expression with that of preproinsulin in insulin-producing beta-TC3 cells. *Biochemical Journal* 313, 259–268.

SEIDAH, N. G., HAMELIN, J., MAMARBACHI, M., DONG, W., TADROS, H., MNIKAY, M., CHRETIEN, M. & DAY, R. (1996). cDNA structure, tissue distribution, and chromosomal localization of rat PC7, a novel mammalian proprotein convertase closest to yeast kexin-like proteinases. *Proceedings of the National Academy of Sciences of the USA* 93, 3388–3393.

SEIDAH, N. G., MARCINKIEWICZ, M., BENJANNET, S., GASPAR, L., BEAUBIEN, G., MATTEI, M. G., LAZURE, C., MBIKAY, M. & CHRETIEN, M. (1991). Cloning and primary sequence of a mouse candiate prohormone convertase PC1 homologous to PC2, furin, and kex2: distinct chromosomal localization and messenger RNA distribution in brain and pituitary compared to PC2. *Molecular Endocrinology* 5, 111–122.

SEVA, C., DICKINSON, C. J. & YAMADA, T. (1994). Growth-promoting effects of glycine-extended progastrin. *Science* 265, 410–412.

SINGH, P., OWLIA, A., ESPEIJO, R. & DAI, B. (1995). Novel gastrin receptors mediate mitogenic effects of gastrin and processing intermediates of gastrin on Swiss 3T3 fibroblasts. *Journal of Biological Chemistry* 270, 8429–8438.

SMEEKENS, S. P., AVRUCH, A. S., LAMENDOLA, J., CHAN, S. J. & STEINER, D. F. (1991). Identification of a cDNA encoding a second putative prohormone convertase related to PC2 in AtT20 cells and islets of Langerhans. *Proceedings of the National Academy of Sciences of the USA* 88, 340–344.

STOFFERS, D. A., GREEN, C. B.-A. & EIPPER, B. A. (1989). Alternative mRNA splicing generates multiple forms of peptidyl-glycine α-amidating monooxygenase in rat atrium. *Proceedings of the National Academy of Sciences of the USA* 86, 735–739.

STOFFERS, D. A., OUAFIK, L'H. & EIPPER, B. A. (1991). Characterization of novel mRNAs encoding enzymes involved in peptide α-amidation. *Journal of Biological Chemistry* 266, 1701–1707.

TODISCO, A., TAKEUCHI, Y., SEVA, C., DICKINSON, C. J. & YAMADA, T. (1995). Gastrin and glycine-extended progastrin processing intermediates induce different programs of early gene activation. *Journal of Biological Chemistry* 270, 28337–28341.

VARRO, A., HENRY, J., VAILLANT, C. & DOCKRAY, G. J. (1994). Discrimination between temperature- and brefeldin A-sensitive steps in the sulfation, phosphorylation, and cleavage of progastrin and its derivatives. *Journal of Biological Chemistry* 269, 20764–20770.

VARRO, A., VORONINA, S. & DOCKRAY, G. J. (1995). Pathways of processing of the gastrin precursor in rat antral mucosa. *Journal of Clinical Investigation* 95, 1642–1649.

VARRO, A., YEGEN, B. & DOCKRAY, G. J. (1993). Control of tissue progastrin concentrations in the rat. *Experimental Physiology* 78, 327–336.

WALDBIESER, G. C., AIMI, J. & DIXON, J. E. (1991). Cloning and characterization of the rat complementary deoxyribonucleic acid and gene encoding the neuroendocrine peptide 7B2. *Endocrinology* 128, 3228–3236.

WALSH, J. H. (1994). Gastrin. In *Gut Peptides*, ed. WALSH, J. H. & DOCKRAY, G. J., pp. 75–121. Raven Press, New York.

WALSH, J. H., DEBAS, H. T. & GROSSMAN, M. I. (1974). Pure human big gastrin: immunochemical properties, disappearance half time and acid stimulating action in dogs. *Journal of Clinical Investigation* 54, 477–485.

WALSH, J. H., ISENBERG, J. I., ANSFIELD, J. & MAXWELL, V. (1976). Clearance and acid-stimulating action of human big and little gastrins in duodenal ulcer subjects. *Journal of Clinical Investigation* 57, 1125–1131.

WALSH, J. H., RICHARDSON, C. T. & FORDTRAN, J. S. (1975). pH dependence of acid secretion and gastrin release in normal and ulcer subjects. *Journal of Clinical Investigation* 55, 462–468.

WANG, T. C., KOH, T. J., VARRO, A., CAHILL, R., DANGLER, C. A., FOX, J. G. & DOCKRAY, G. J. (1996). Processing and proliferative effects of human progastrin in transgenic mice. *Journal of Clinical Investigation* 98, 1918–1929.

WU, S. V., GIRAUD, A., MOGARD, M., SUNII, K. & WALSH, J. H. (1990). Effects of inhibition of gastric secretion on antral gastrin and somatostatin gene expression in rats. *American Journal of Physiology* 258, G788–793.

ZHU, W. & LINDBERG, I. (1995). 7B2 facilitates the maturation of proPC2 in neuroendocrine cells and is required for the expression of enzymatic activity. *Journal of Cell Biology* 129, 1641–1650.

Author's email address

A. Varro: avarro@liverpool.ac.uk