Transcriptomes of purified gastric ECL and parietal cells: identification of a novel pathway regulating acid secretion

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Lambrecht, Nils W. G., Iskandar Yakubov, Cindy Zer, and George Sachs. Transcriptomes of purified gastric ECL and parietal cells: identification of a novel pathway regulating acid secretion. Physiol Genomics 25: 153–165, 2006. First published January 10, 2006; doi:10.1152/physiolgenomics.00271.2005.—The gastric entero-chromaffin-like (ECL) cell plays a key regulatory role in peripheral regulation of acid secretion due to the release of histamine that stimulates acid secretion by the parietal cell. Studies in intact animals, gastric glands, and isolated cells after short-term culture have shown expression of stimulatory CCK2 and PAC1 and inhibitory SST2 and Gal1 receptors as well as histidine decarboxylase. However, the pattern of its gene expression as a neuroendocrine cell has not been explored. Comparison of gene expression by 95% pure ECL cells obtained by density gradient, elutriation, and fluorescence-assisted cell sorting with isolates of the intact fundic gastric epithelium (i.e., “subtractive hybridization”) identified a variety of additional expressed gene families characteristic of this neuroendocrine cell. These include genes 1) involved in neuropeptide synthesis and secretory vesicle exocytosis, 2) involved in control of inflammation, 3) implicated in healing of the epithelium, 4) encoding inhibitory G protein-coupled receptors, 5) playing a role in neuroendocrine regulation of food intake, and 6) encoding proteins likely involved in maintenance of circadian rhythm, in addition to the ECL cell-specific genes histidine decarboxylase and monoamine transporter. Particularly, the inhibitory apelin receptor gene, APJ, was highly expressed in the ECL cell preparation. Because parietal cells express apelin, immunohistochemical and functional studies showed that there is an inhibitory feed back loop between the parietal and ECL cell during gastrin stimulation, providing evidence for a novel pathway of downregulation of acid secretion due to interaction between these two cell types.

entero-chromaffin-like cell; oligonucleotide expression microarray; transcriptome; apelin; APJ receptor

THE GASTRIC FUNDIC ENTERO-CHROMAFFIN-LIKE (ECL) cell is the major neuroendocrine cell of the gastric oxyntic mucosa. Its known function is to regulate the peripheral phase of acid secretion by the parietal cell. ECL cells have been extensively studied over the past decade with the advent of techniques for isolation, enrichment, and functional assessment. An unexpected finding with these isolated cells was the presence and activity of the pituitary adenylate cyclase-activating peptide (PACAP) receptor 1 (PAC1) and absence of a cholinergic receptor, indicating that PACAP, not acetylcholine, was the neural mediator of regulation of ECL cell function in stimulation of acid secretion (1, 21, 68). Various inhibitory receptors such as the somatostatin receptor subtype 2 (SSTR2) (42) and the galanin 1 (Gall) receptor (69) were also identified. Although these findings provided insight into the factors that regulate activity of ECL cells, the techniques utilized do not allow a full description of all the functional properties of these cells.

The major pathways of direct activation of parietal cells are histamine stimulation of the H2 receptor and acetylcholine activation of the M3 muscarinic receptor (72). The hormone gastrin, released from antral G cells due to stimulation by aromatic amino acids, and the neuropeptide PACAP, released from local vagal nerve endings, act indirectly by releasing histamine from ECL cells (43, 68). The sympathetic agonists adrenaline, isoprenaline, and terbutaline also stimulate acid secretion by activating a β2-receptor subtype on ECL cells to release histamine (41) as well as directly on parietal cells (67). Known inhibitory pathways of gastric acid secretion are mediated by somatostatin, released from D cells, via binding to SSTR2, the somatostatin receptor subtype present on parietal cells, ECL cells, G cells, and neurons of the myenteric plexus; and by the neuropeptide galanin via the Gall receptor on ECL cells (2, 19, 42, 69). A direct negative-feedback mechanism on histamine release was proposed because of the presence of an inhibitory histamine 3 receptor subtype on ECL cells (23, 41, 54). This has not been conclusively demonstrated to have physiological relevance, since histamine is rapidly degraded. ECL cells also express the PYY Y1 receptor, which, on isolated ECL cells, shows inhibition of gastrin-stimulated histamine release (70). However, in situ microdialysis studies showed slight elevation of histamine release in the rat stomach on application of PYY (38). The basis for these controversial results remains to be elucidated but may involve the presence of PYY receptors on cells other than ECL cells, such as the G cell. This is reminiscent of controversial data on PACAP stimulation of isolated ECL cells but PACAP inhibition of secretion in the animal unless somatostatin antibody is also administered. These data were explained by the presence of a PAC1 receptor on the ECL cell and a vasointestinal peptide (VIP) receptor on the D cell that stimulated somatostatin release (68), where the latter was stimulated by the injected peptide, which may not occur physiologically where PACAP is released locally in the environment of the ECL cell from vagal nerves.

To better understand other cellular functions of ECL cells, a detailed gene expression profile was performed. We used our recently introduced oligonucleotide expression microarray-based approach that we have termed “subtractive hybridization.” This method allows us to obtain an mRNA expression profile of specific cell types of the gastric mucosa by compar-

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The text continues with further details and scientific findings related to the ECL cell and its role in acid secretion. It discusses various pathways and receptors involved in the regulation of acid secretion, including histamine, gastrin, and various neuropeptides. The text also addresses the limitations of current research methods in understanding the full range of ECL cell function.
ing the gene expression of a highly purified single cell type with the gene expression of the whole gastric fundic epithelium (27). The present work describes a novel technique for purification of gastric ECL cells to >95% purity and then compares their mRNA expression profile with the profile of all cells of the gastric mucosal epithelium and with a previously defined profile of homogeneous (>99%) parietal cells. We were able to recognize a significant number of previously undescribed genes in ECL cells allowing us to define novel functional properties of ECL cells, including control of inflammation, wound healing, epithelial regeneration, hormonal regulation of food intake, and control of circadian rhythm.

Specific expression of the neuropeptide apelin in parietal cells (27) and apelin receptor (APJ-R) in ECL cells prompted us to further assess whether this receptor-neuropeptide pair could play an inhibitory role in the peripheral phase of acid secretion. We were able to show 1) apelin inhibition of gastrin-stimulated intracellular Ca\(^{2+}\) elevation in ECL cells, 2) apelin inhibition of gastrin- but not histamine-stimulated acid secretion in intact rabbit gastric glands, and 3) immunohistochemical localization of the apelin receptor on ECL cells and apelin-containing granules in parietal cells. We postulate that the apelin-APC receptor pair represents a direct negative-feedback regulation of parietal cells on ECL cell histamine release during acid secretion that has not been previously described.

**EXPERIMENTAL PROCEDURES**

**ECL Cell Purification from Rat Stomach**

All animal studies have been approved (protocol no. 05037-04) by the Veterans Affairs Greater Los Angeles Healthcare System Animal Research Committee. Primary gastric mucosal digests were prepared as described previously with slight modifications (27). Briefly, four to five stomachs from 200- to 250-g male Sprague-Dawley rats were used to yield 80 ml of primary mucosal digested cell suspensions in buffer C (140 mM NaCl, 1.2 mM MgSO\(_4\), 1 mM CaCl\(_2\), 10 mM HEPEs, 11 mM glucose, and 0.5 g/l BSA, pH 7.4) containing 300 mg/l DTT and 10 mM SCH28080. Cells were washed twice in solution C with 10\(^{-4}\)M SCH28080 and concentrated to a total volume of 5 ml in solution C containing 300 mg/l DTT and 10 \(\mu\)M SCH28080.

*Fluorescence-Assisted Cell Sorting*

ECL cells accumulate acridine orange [acidic dissociation constant (pK\(_a\)) ~ 9.0] in their histamine-containing vesicles due to the acidic pH of 5–5.5 generated by the activity of a V-type ATPase that is insensitive to inhibition by SCH28080 at concentrations <100 \(\mu\)M (44). At this pH and dye concentration, the ECL cell histamine-containing vacuoles show combined red and green fluorescent emission resulting in a yellow fluorescent appearance of ECL cells at an excitation wavelength of 488 nm. This fluorescence profile is explained by the fact that about one-half of the dye is sufficiently stacked to generate a red chromatic shift (red emission) and one-half of the dye, although accumulated, still shows green emission.

Therefore, acridine orange (100 nM) was added to the ECL suspensions as prepared above, and the labeled cells were sorted using the University of California Los Angeles Flow Cytometry Core and the FACStar PLUS service. Excitation wavelength was 488 nm; sorting was achieved by gating cells showing emission at both 532 and 585 nm. Cells were sorted over a 4-h time period, pooled in solution C containing 10 units of RNase inhibitor (Promega), and pelleted by centrifugation in a Sorvall centrifuge for 5 min at 1,100 rpm. The majority of cells were resuspended in 100–200 \(\mu\)l of RNAlater (Qiagen) to inhibit RNase activity.

*cRNA Labeling and Rat Expression Oligonucleotide Microarray Hybridization*

Freshly resected rat stomachs were opened, and the oxyntic mucosa was scraped off the stomach wall by using the sharp edge of a slide. The fragments were filtered through a nylon sieve in 1.5 ml of RNAlater (Qiagen) and homogenized on ice. Total RNA from this homogenate (ST) or from cell suspensions obtained from the fluorescence-assisted cell sorting (FACS) P2 fraction (see RESULTS AND DISCUSSION below) was isolated using a NucleoSpin RNA II Kit (BD Biosciences). Typical RNA concentrations were 200–300 ng/\(\mu\)l. The RNA was assessed for purity and stability using a Bioanalyzer 2100 (Agilent Technologies). These preparations represent either total gene expression by the gastric epithelium or the ECL cell fraction.

Fluorescently labeled cRNA was generated using an RT reaction with a poly (dT)-T7 promoter primer followed by T7 polymerase-based linear amplification in the presence of fluorophor-labeled nucleotides Cy3- or Cy5-CTP according to the manufacturer’s protocol (Low RNA Input Fluor Linear Amp Kit, Agilent Technologies). The final cRNA concentration of typically 300–500 ng/\(\mu\)l and the Cy3- or Cy5-cytidine incorporation of 5–10 pmol/\(\mu\)g cRNA were determined using a Nanodrop spectrophotometer ND-1000 (Nanodrop Technologies); 3.5 \(\mu\)g of labeled cRNA from whole gastric mucosal epithelium were combined with labeled cRNA from each purified cell suspension and hybridized to a 22K rat oligonucleotide expression array (Agilent Technologies) according to the manufacturer’s protocol. All three microarray experiments described here contained one dye swap experiment. All microarrays were scanned, and the intensities were normalized over background using a microarray scanner from Agilent Technologies including proprietary software. The microarray data were deposited for public access to the Gene Expression Omnibus National Center for Biotechnology Information (NCBI) database under accession numbers GSE3518, GSM80287, and GSM80288.

*Immunohistochemical Studies*

*Rat gastric paraffin-embedded sections.* Stomachs of male Sprague-Dawley rats, weighing 200–300 g, were removed, washed, and immediately fixed in 10% formaldehyde overnight. The stomachs were sectioned and embedded in paraffin, and 3-\(\mu\)m sections were serially cut using standard histology methods. Slides with tissue sections were deparaffinized (3 \(\times\) 100% xylene, 5 min) and rehydrated (1 \(\times\) 100% ethanol, 1 \(\times\) 95% ethanol, 1 \(\times\) 85% ethanol, 1 \(\times\) 70% ethanol, 5 min each). Sections were rinsed twice in PBS, immersed in antigen retrieval buffer (0.01 M sodium citrate buffer, pH 6) and microwaved for 2 min (600 W), reaching boiling temperature. Sections were rinsed twice in PBS and incubated with protein-free blocking solution (DAKO) for 30 min. Polyclonal antibody (rabbit) against apelin (Phoenix Pharmaceuticals) and monoclonal antibody
against H⁺-K⁺-ATPase (12.18) were added in a 1:100 dilution in PBS and incubated for 1 h. Control slides were incubated with 10% normal goat serum in PBS (Life Technologies) instead of the primary antibody. Slides were washed for 15 min in PBS and incubated for 30 min in a solution containing anti-rabbit TRITC and anti-mouse FITC conjugates (American Qualex, 1:100 in PBS). The slides were finally washed for 15 min in PBS and mounted in 70% glycerol in PBS.

**Rabbit gastric glands.** Glands were prepared as described below by standard methods. Glands were fixed for 15 min at room temperature on glass slides in Tris-buffered solution (TBS; 20 mM Tris, 150 mM NaCl, pH 7.4) containing 3.7% paraformaldehyde. The slides were washed twice in TBS-Tween (TBS containing 0.2% Tween 20) and incubated for 30 min at room temperature in TBS-Tween containing 10 mg/ml bovine serum albumin (TBS-Tween-BSA) with 0.7% Triton X-100 added for permeabilization of the gland membranes. The slides were washed twice more in TBS-Tween and incubated for 1 h at room temperature with a 1:100 dilution of the primary antibody [anti-histidine decarboxylase (HDC) polyclonal antibody (Eurodiagnostics, Malmö, Sweden) or anti-APJ receptor monoclonal antibody (Neuromics) in TBS-Tween-BSA]. Control slides were incubated with 10% normal goat serum in TBS-Tween-BSA (Life Technologies) instead of the primary antibody. The slides were washed twice in TBS-Tween and incubated in TBS-Tween-BSA containing a 1:100 dilution of the secondary antibody against mouse or rabbit IgG Fc region conjugated either with FITC or TRITC as needed. The slides were washed again for 15 min in TBS-Tween and mounted in 70% glycerol in PBS.

**Confocal microscopy.** The slides were placed under a confocal microscope (Zeiss, LSM510) that was set up using multitrack mode for 15 min in TBS-Tween and mounted in 70% glycerol in PBS. The slides were placed into an orbital shaker and incubated for 30 min at 37°C at 150 rpm. The glands were pelleted using a microcentrifuge at 10,000 rpm for 1 min; 800 µl of supernatant were removed and counted in 10 ml of scintillation fluid. The remainder of the supernatant was carefully removed, the wet tube plus pellet were weighed, and the tubes were dried at 37°C overnight. The dry tubes plus pellet were weighed. 100 µl of 1 N NaOH were added, and the tubes were incubated at 37°C for 3 h. This completely dissolves the dry pellet. The tips of the tubes were cut off, immersed in 10 ml of scintillation fluid, and counted. The 14C-aminopyrine uptake ratio was obtained by dividing the corrected counts per minute of the pellet (trapped 14C-aminopyrine) by the counts per minute of the supernatant. All experiments were performed in triplicate, and the means ± SD were plotted.

**Intracellular Calcium Imaging in Rabbit Gastric Glands**

Freshly isolated gastric glands were loaded with 5 µM Fluor4/AM (Molecular Probes) for 15 min at 37°C in the incubator. The last 5 min of incubation were performed in CellTak (Collaborative Biomedical Products)-coated glass bottom petri dishes (MatTek) to allow attachment of the glands. The petri dish was placed under a confocal microscope (Zeiss, LSM510) using a heated stage at 37°C and constant superfusion of the cells at a pump speed of 8 ml/min to avoid cell cross talk. Excitation was 488 nm, and the emission signal was recorded after passage through a 505-nm long-pass filter. The glands were superfused with media (140 mM NaCl, 1.2 mM MgSO₄, 1 mM CaCl₂, 10 mM HEPES, 11.1 mM d-glucose, and 0.2% BSA) containing 1 or 10 nM gastrin I in the absence or presence of 100 nM apelin. Images were recorded every 5 s, and the change of fluorescent intensity was measured in several cells of interest (region of interest; ROI).

**RESULTS AND DISCUSSION**

**Enrichment of Gastric ECL Cells to 95% Purity**

Description of all the genes and their encoded proteins expressed in the ECL cell has been difficult to achieve up to now because this cell represents only 2–3% of the gastric fundic cell population and is diffusely located within the gastric fundic epithelium. Use of cells cultured for a short time is problematic because this is likely to cause changes in gene expression. Thus the development of a purification strategy and the availability of >95% pure ECL cell suspensions without short-term culture represent a prerequisite en route to a physiologically relevant gene expression profile of this important cell. We obtained 40–50% ECL cell-enriched cell suspensions using a combination of 1) density centrifugation (12% nycodenz) and 2) counterflow elutriation (small- to medium-sized cells). Most of the contaminating cells represent small parietal cells (PC) as seen in the confocal image of Fig. 1.

The cells were loaded with 100 nM acridine orange (AO), a membrane-permeable weak base that has unique metachromatic emission characteristics depending on the degree of acidity and hence stacking of the accumulated dye. ECL cells show the typical accumulation of AO in moderately acidic compartments (pH ~ 5). Here the dye at this medium concentration is 50% stacked (TRITC-type emission, red pseudo-
color), and 50% is nonstacked although accumulated (FITC-type emission, green pseudocolor), resulting in the bright yellow appearance of histamine-containing vesicles, which are mostly found on one side of the cell. In contrast, PC are seen to contain bright red (TRITC emission only) punctuate acidic spaces consistent with highly acidic compartments within these cells. This difference in the emission spectrum of AO in PC compared with ECL cells was used to define the sorting parameters for FACS.

Figure 2 shows the flow analysis chart of the above cell suspension and the placement of gates P1 (PC) and P2 (ECL cells). Typically, 1–3 million cells were sorted in each gate. The P1 (PC) and P2 (ECL) cell fractions were analyzed using AO and confocal microscopy. Quantitative analysis of the P2 fraction showed 90–95% cells exhibiting the typical yellow color of AO accumulation of weak acidic granules consistent with identification of ECL cells (Fig. 3).

**mRNA Expression Analysis of Gastric ECL Cells**

Gene expression profiling is a relatively new and powerful method to elucidate cell-specific protein expression and to identify new target proteins important for specific cell functions. Subtractive hybridization (27), the procedure by which we subtract commonly expressed genes in all cell types of the gastric mucosa from the analysis using the purification factor of the purified ECL cell suspension, allows us to determine ECL cell-specific gene expression. Furthermore, comparison of this expression profile with the expression profile of PC (27) is an even more powerful mechanism to ascertain significant and specific ECL cell gene expression. This method shows genes that are expressed in most gastric epithelial cells (ratio of ∼1 in both ECL/St and PC/St), genes that are specific to ECL cells (ratio >3 in ECL/St, <1 in PC/St), and genes that have high expression in ECL cells but in other cell types of the mucosa as well (ratio of 1–3 in ECL/St). Figure 4 shows a typical composite image of the signal of hybridized ECL cell cRNA (green) and gastric mucosal cRNA (ST, red).

We performed three independent experiments, each containing one dye swap analysis. The average standard deviation of the three ratios of expression intensity of the ECL cell sample compared with whole gastric epithelium (ECL/St) was 1.05. The quality of the array data was also assessed by analysis of expression ratios of commonly used housekeeping genes and
known ECL cell-specific genes compared with whole gastric epithelium or homogenously purified PC as published previously (27).

This technique enabled not only confirmation of expression of most marker genes known in the literature to be present in ECL cells, including HDC and vesicular monoamine transporter 2 (VMAT2) as well as cholecystokinin (CCK)B/gastrin, SSRT2/somatostatin, and Gal1/galanin receptor genes as shown in Table 1, but greatly expanded the gene profile of this important neuroendocrine cell. The latter two receptors appear to be expressed not only in ECL cells but also in other cell types in the stomach, since mRNA expression was also high in whole gastric mucosal RNA compared with other tissues (data not shown). CCKB/gastrin receptor expression was confirmed to be present in PC in addition to ECL cells (Table 1) as previously functionally identified (3).

Table 1 also shows that a small number of X/A (ghrelin) and G (gastrin) cells are present in the preparation, given the slight enrichment of these genes, but that D (somatostatin) cells are depleted. Genes for secreted proteins are usually very highly expressed, and this enrichment overestimates the contaminating percentage of these X/A and G cells. It should also be noted that, since PC constitute 25–35% of the gastric fundic cell population, the enrichment of selective gene markers for this cell type is in the range of two- to threefold in contrast to the higher expression elevation for markers for ECL cells, which constitute no more than 2–3% of the rat gastric fundic cells.

The analysis presented here confirms the high degree of purity of ECL cells in the P2 cell suspension assayed (P2, Fig. 3) on an mRNA expression level.

Global Gene Expression Profile of Gastric ECL Cells

We identified 448 genes that were significantly highly expressed by ECL cells but not PC or other cells of the gastric epithelium from a total of 20,501 unique genes present on this rat microarray platform. These genes showed an increased message level of at least threefold in the ECL cell mRNA messages compared with whole gastric epithelium or at least two times the average standard deviation of all ratios on the array \( P \leq 0.05, \) confidence interval (CI) = 95% (1 + 2.1 = 3.1]), but with a concurrent decrease of the same genes in RNA samples from PC.

ECL cell-specific mRNA messages include genes coding for proteins involved in cell membrane transport processes (16 genes), vesicular transport (7 genes), cell-to-cell contact formation (7 genes), cytoskeletal structure and fluidity (18 genes), receptors (21 genes), receptor-associated proteins (6 genes), signal transduction (7 genes), enzymes (11 genes) and proteases (4 genes), transcription factors (22 genes) and secreted...
peptides/growth factors (16 genes). The expressed genes can be clustered in at least six major functional groups: 1) genes involved in neuropeptide synthesis and secretory vesicle exocytosis, 2) genes involved in control of inflammation, 3) genes implicated in healing of the epithelium, 4) genes encoding for inhibitory G protein-coupled receptors, 5) genes that play a role in neuroendocrine regulation of food intake, and 6) genes involved in regulation of the circadian rhythm. The expression profile supports the hypothesis that the ECL cell is a neuroendocrine cell involved not only in histamine release and regulation of acid secretion but also in facilitating neuroendocrine cell secretion and regulation and local anti-inflammatory control as well as cell growth and differentiation of the gastric epithelium. These findings broaden our understanding of the role of the ECL cell in gastric function.

The following sections first discuss genes likely to indicate new functional characteristics of ECL cells, and then we focus on the discovery of a novel negative-feedback pathway between parietal and ECL cells. The expression profile also presents a database of other possibly functionally important target genes that can be tested for their significance in existing experimental models of gastric physiology. Hence, some of the most specifically expressed genes of ECL cells are listed in Table 2 and are further discussed below.

**Genes implicated in neuroendocrine secretion.** The expression profile identified 19 transcripts coding for proteins with known functions that are involved in vesicular transport, exocytosis, and peptide processing of neuroendocrine mediators.

The main mediator of ECL cells in the regulation of acid secretion is histamine. Histamine is synthesized via HDC and accumulated in specialized secretory vesicles via VMAT2 (43), both found to be highly expressed in the ECL cell preparation (Table 1). The proton gradient driving VMAT2 histamine uptake is generated via the V-type ATPase, several subunits of which are highly expressed in ECL cells (data not shown). However, only a twofold increase in expression of these subunits compared with gastric mucosal cells was detected, indicating V-type ATPase expression in other cell types, as would be expected in lysosomes and perhaps other acidic secretory vesicles and even in chief cells.

Neuroendocrine cells may contain two separate pools of vesicles, large secretory granules and small synaptic-like microvesicles (24). The presence of specific genes encoding chromogranin A and B but not synaptophysin (data not shown) indicates the presence of mostly genes involved in exocytosis of large secretory granules by ECL cells.

Both types of vesicles are transported to the plasma membrane by cytoskeletal motor proteins (25), are tethered to the membrane by Rab-GTPases, physically docked by formation of the SNARE complex and the mediator released by membrane fusion regulated by Ca \(^{2+}\) binding to synaptotagminis (for review, see Refs. 56 and 60). ECL cells specifically express kinesin 12 (vesicular transport), Rab GTPase 3c (but not Rab3a, -b, or -d; data not shown), Doc2B and the Munc13 homolog Cadps (vesicle tethering and t-snare activation; Refs. 16, 59), SNAP 25 [a t-snare that forms together with syntaxin3a (t-snare) and synaptobrevin (v-snare), the SNARE vesicle-plasma membrane docking complex; Ref. 56]; and synaptotagmin 7 (Ca \(^{2+}\) sensor) to stimulate membrane fusion (60). The expression of these genes is expected to be present in the ECL cell, the function of which is mainly subserved by exocytosis. Syntaxin 3a and synaptobrevin are only 2.3-fold higher expressed in ECL cells than in whole gastric epithelium, indicating expression of these genes in other cell types of the gastric mucosa as well, for example again in other endocrine cells and chief cells.

Another finding is the highly specific expression of synaptic vesicle 2-related protein (SVOP) but not synaptic vesicle protein (SV)-2a, -b, or -c (data not shown) in ECL cells. SVOP exhibits similarity (20–22% sequence identity) in general protein topology with SV2 proteins (both contain 12 putative transmembrane helices) but, in contrast to SV2 proteins, is nonglycosylated. Although the function of SVOP is presently unknown, homology searches identified that the closest relatives are organic anion or cation transporters (24). Previous studies in adrenal chromaffin cells colocalized SVOP to the microsomal compartment (small synaptic-like vesicles) and SV2 to large secretory granules (24). However, ECL cells express neuroendocrine vesicular proteins (Rab3e, synaptotagmin) in relatively high amounts, similar to large “chromaffin”-like secretory vesicles, which are seen in our confocal immunofluorescent images of ECL cells, the vesicles of which are >100 nm in diameter. There does not appear to be much small synaptic-like vesicle protein expression in the ECL cell gene profile except for SVOP (Rab3a, synaptophysin, and synapsins). This further supports the notion that ECL cells have specialized “chromaffin-like” vesicles with slightly different protein composition compared with other neuroendocrine cells.

There was also specific expression of carboxypeptidase E as well as pro-protein convertase subtilisin/kexin type 2 and its regulatory chaperone, secretory granule neuroendocrine protein 1 (34). These proteins are involved in regulated neuroendocrine peptide processing from protein precursors like chromogranins A (11) and -B (4) and vgf (30) in secretory granules. This indicates that ECL cells not only synthesize and release histamine but also other neuroendocrine peptides like pancreastatin, a peptide derived from chromogranin A (71) that inhibits both endo- and exocrine pancreatic function (12, 15). Of interest here is also the highly specific expression of

<table>
<thead>
<tr>
<th>Description</th>
<th>Predicted Cell Type</th>
<th>ECL/St</th>
<th>PC/St</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (Gapd)</td>
<td>All</td>
<td>1.06</td>
<td>1.26</td>
</tr>
<tr>
<td>Na(^+), K(^+) ATPase alpha 1 (Atplal1)</td>
<td>All</td>
<td>0.73</td>
<td>1.21</td>
</tr>
<tr>
<td>Histidine decarboxylase (Hdc)</td>
<td>ECL</td>
<td>13.65</td>
<td>0.14</td>
</tr>
<tr>
<td>Vesicular monoamine transporter 2 (Slc18a2)</td>
<td>ECL</td>
<td>8.70</td>
<td>0.54</td>
</tr>
<tr>
<td>Gastrin (Gas)</td>
<td>G</td>
<td>1.97</td>
<td>0.33</td>
</tr>
<tr>
<td>Somatostatin (Sst)</td>
<td>D</td>
<td>0.58</td>
<td>0.09</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>X/A</td>
<td>1.77</td>
<td>0.14</td>
</tr>
<tr>
<td>Pepsinogen F</td>
<td>Chief</td>
<td>0.37</td>
<td>0.10</td>
</tr>
<tr>
<td>Mucin 1 (Muc1)</td>
<td>Mucus</td>
<td>0.50</td>
<td>0.03</td>
</tr>
<tr>
<td>Kenk</td>
<td>Parietal</td>
<td>0.63</td>
<td>4.63</td>
</tr>
<tr>
<td>Ezrin</td>
<td>Parietal</td>
<td>0.77</td>
<td>3.02</td>
</tr>
<tr>
<td>Cholecystokinin B/gastrin receptor (Cckbr)</td>
<td>ECL + parietal cell</td>
<td>2.18</td>
<td>1.34</td>
</tr>
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</table>

ECL cell, entero-chromaffin-like cell; G cell, gastrin cell; D cell, somatostatin cell; X/A cell, ghrelin cell.
Table 2. Expression ratios of selected ECL cell-expressed genes (ECL) and purified parietal cells (PC) compared with total gastric fundic epithelium genes (St)

<table>
<thead>
<tr>
<th>Description</th>
<th>Putative Function</th>
<th>ECL/St</th>
<th>PC/St</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neuroendocrine secretion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kinesin family member 12 (Kif12)</td>
<td>Vesicular transport</td>
<td>8.65</td>
<td>0.58</td>
</tr>
<tr>
<td>Rab3c GTPase</td>
<td>Vesicle tethering</td>
<td>9.83</td>
<td>0.47</td>
</tr>
<tr>
<td>Double C2, β (Doc2B)</td>
<td>t-SNARE activation</td>
<td>8.14</td>
<td>0.91</td>
</tr>
<tr>
<td>Ca^{2+}-dependent activator protein for secretion (Cadps)</td>
<td>t-SNARE activation</td>
<td>5.41</td>
<td>0.38</td>
</tr>
<tr>
<td>VAMP-1/synaptobrevin</td>
<td>t-SNARE</td>
<td>2.31</td>
<td>0.58</td>
</tr>
<tr>
<td>Snap25, t-snare</td>
<td>t-SNARE</td>
<td>7.16</td>
<td>0.35</td>
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<tr>
<td>Syntaxin 3a, t-snare</td>
<td>t-SNARE</td>
<td>2.32</td>
<td>0.66</td>
</tr>
<tr>
<td>Synaptogamin 7 (Syn7)</td>
<td>Control of exocytosis (membrane fusion)</td>
<td>5.76</td>
<td>0.49</td>
</tr>
<tr>
<td>Carboxypeptidase E (Cpe)</td>
<td>Secretory peptide processing</td>
<td>15.50</td>
<td>0.14</td>
</tr>
<tr>
<td>Secretory granule neuroendocrine protein 1 (Sgne1)</td>
<td>Secretory peptide processing</td>
<td>9.23</td>
<td>0.53</td>
</tr>
<tr>
<td>Proprotein convertase subtilisin/kexin type 2 (Pcsk2)</td>
<td>Secretory peptide processing</td>
<td>4.76</td>
<td>0.58</td>
</tr>
<tr>
<td>Regulated endocrine-specific protein 18 (Resp18)</td>
<td>Transcriptional control of neuropeptide synthesis</td>
<td>15.26</td>
<td>0.24</td>
</tr>
<tr>
<td>Chromogranin B, parathyroid secretory protein (Chgb)</td>
<td>Neuropeptide precursor</td>
<td>9.96</td>
<td>0.19</td>
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<tr>
<td>Chromogranin A, parathyroid secretory protein 1 (Chga)</td>
<td>Neuropeptide precursor</td>
<td>5.78</td>
<td>0.21</td>
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<tr>
<td>SVOP (SV2-related protein)</td>
<td>Unknown</td>
<td>13.16</td>
<td>0.51</td>
</tr>
<tr>
<td><strong>Inflammation and healing</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Murinoglobulin 1 [Alpha(1)-inhibitor 3]</td>
<td>Unspecific protease inhibition</td>
<td>22.85</td>
<td>0.09</td>
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<tr>
<td>C1-esterase inhibitor (Serpina1)</td>
<td>Complement inhibition</td>
<td>7.41</td>
<td>0.26</td>
</tr>
<tr>
<td>Thrombomodulin (Thbd)</td>
<td>Thrombin inhibition</td>
<td>13.88</td>
<td>0.06</td>
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<tr>
<td>Vitronecrotin (Vtn)</td>
<td>Thrombin inhibition and healing (extracellular matrix)</td>
<td>7.08</td>
<td>0.53</td>
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<tr>
<td>Lipostatin, pancreatic stone protein (Reg1)</td>
<td>Cell growth and differentiation</td>
<td>22.69</td>
<td>0.31</td>
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<tr>
<td>NOV (nephroblastoma overexpressed gene, CCN3)</td>
<td>Cell growth and differentiation</td>
<td>16.07</td>
<td>0.62</td>
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<tr>
<td><strong>Receptors and associated proteins</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid receptor PUMA-G (HM74a/b, Gpr109a)</td>
<td>Nicotinic acid binding, inhibition of lipolysis</td>
<td>13.05</td>
<td>0.90</td>
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<tr>
<td>APJ receptor (APJ, Agt11)</td>
<td>Apelin binding, inhibition of acid secretion</td>
<td>8.22</td>
<td>0.66</td>
</tr>
<tr>
<td>GABA B1 receptor (GABBR1)</td>
<td>GABA binding, stimulation of acid secretion</td>
<td>5.84</td>
<td>0.98</td>
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<tr>
<td>Activating transcription factor ATF-4 (Atf4)</td>
<td>Transcription regulation through GABA-B receptor</td>
<td>6.38</td>
<td>0.68</td>
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<td>Low density lipoprotein receptor-related protein 11 (Lrp11)</td>
<td>Unknown</td>
<td>5.66</td>
<td>1.06</td>
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<td>Guanylate cyclase 2C (heat stable enterotoxin) receptor (Gucy2c)</td>
<td>Lrp11 receptor regulation</td>
<td>7.19</td>
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<td></td>
<td>Guanylin binding, regulation of water transport</td>
<td>5.56</td>
<td>1.01</td>
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<tr>
<td><strong>Other neuroendocrine regulatory functions</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>VGF neuroendocrine protein</td>
<td>Regulation of energy expenditure and feeding</td>
<td>24.40</td>
<td>0.82</td>
</tr>
<tr>
<td>Period circadian protein 1 (Per1)</td>
<td>Regulation of circadian rhythm</td>
<td>2.92</td>
<td>1.01</td>
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<tr>
<td>Gpraspl/PIPS (G protein-coupled receptor-associated sorting protein/Per1-interacting protein)</td>
<td>Regulation of circadian rhythm</td>
<td>8.15</td>
<td>1.34</td>
</tr>
</tbody>
</table>

Resp18, a transcriptional regulator thought to sense neuropeptide synthesis in vesicles of neuroendocrine cells and then regulate transcription of proteins involved in this process (8, 49). Some of the specifically expressed genes identified here using gene expression analysis have also been found in an elegant immunohistochemical localization study on human gastric mucosal sections (22).

**Genes implicated in local control of inflammation.** During acute injury, generation of thrombin and complement factors contribute not only to the initial blood coagulation cascade and defense to the offending agent but also to the damage of noninjured cells. Therefore, it is prudent for the body to protect cells against the action of these proteases. The expression profile of ECL cells showed highly specific expression of messages coding for a C1-esterase inhibitor (6) that inhibits the complement cascade as well as thrombomodulin (14), vitronectin (via plasminogen activator inhibitor-1; Ref. 20), and murinoglobulin I (45), which inhibit thrombin and other extracellular pro-inflammatory proteases. These findings suggest that ECL cells perhaps have a gastro-protective function as well as a secretory function.

**Genes involved in epithelial regeneration.** The ECL gene profile showed high expression of a variety of genes encoding peptides important for cell growth and differentiation, an important mechanism for regeneration of the epithelium and wound healing after acute injury due to peptic ulcer or non-steroidal anti-inflammatory drug (NSAID) damage. These include Reg1 and NOV. Reg1 (regenerating gene 1), originally identified in pancreatic secretions as a stone formation inhibitor, lithostatin, was also shown to be expressed in regenerating β-cells of the pancreatic islets. Subsequent studies showed that Reg1 is highly expressed in gastric ECL cells. Expression of this peptide is upregulated in hypergastrinemia, and Reg1 overexpression leads to a significant increase in numbers of parietal and chief cells in a transgenic mouse model. It has also been shown that Reg1 is also highly expressed in inflammatory conditions such as *Helicobacter pylori* infection and during water immersion-induced gastric mucosal erosions (for a review, see Ref. 26). NOV (nephroblastoma overexpressed), also called CCN3 (cysteine-rich/connective tissue growth factor/ nephroblastoma overexpressed), has been implicated in up-regulation of plasminogen activator inhibitor-1 and in wound healing (31). Hence the expression profile of ECL cells suggests that this cell is involved in gastric epithelial repair.

**Receptor expression in ECL cells.** ECL cells are stimulated via CCKB/gastrin and PACAP1 receptors to release histamine.
The latter gene is not represented as an oligonucleotide probe on our 22K rat gene expression microarray. CCKB/gastrin receptor expression has been found in a variety of other gastric mucosal cell types including PC (3, 55), chief cells (63), and D cells (10, 55). This ubiquitous presence is confirmed by the finding that the expression of the receptor mRNA in ECL cells is only 2.2 times higher compared with total fundic epithelium and actually is also slightly more expressed in PC-enriched fractions.

The most specific and highly expressed receptors in the ECL cell gene profile are two inhibitory Gi protein-coupled receptors, the nicotinic acid receptor (PUMA-G, Gpr109a, HM74a/b) and the apelin-binding receptor (APJ, Agtr1l). The former receptor inhibits adenylate cyclase on nicotinic acid binding in adipocytes and subsequent lipolysis and is thought to be the target for acipimox, an anti-hyperlipidemic drug (53). The latter binds apelin and is present in the hypothalamic nuclei (paraventricular and supraoptic nuclei; PVN and SON, respectively), indicating a possible role in mediating neuroendocrine responses of the central nervous system including inhibition of food intake (39, 61). Apelin has been shown to stimulate release of CCK from intestinal neuroendocrine cells (64, 66). The ligands for these two receptors could significantly inhibit histamine release in ECL cells and downregulate acid secretion, and this interesting possibility is discussed in more detail below.

Unexpectedly, specific expression of the metabotropic GABA-B receptor-1 subunit and its transcription factor activating transcription factor-4 (ATF-4) (cAMP-responsive element-binding protein-2; CREB-2) was also found. This receptor controls the transcription of GABA-B receptor subunits-1 and -2 (58) to form fully assembled GABA-B receptor. The GABA-B receptor-2 subunit is not present on the present microarray, and further studies have to be performed using the next generation of gene expression microarrays containing the complete rat genome. However, the fully assembled GABA-B receptor has been shown to inhibit adenylate cyclase as well as voltage-gated calcium channels and could represent a third receptor on ECL cells inhibiting histamine release and acid secretion (32).

The ECL gene profile also shows significant and specific expression of two other receptors, the guanylin-binding heat-stable enterotoxin receptor and the LDL-related receptor-related protein 11 (Lrp11) together with its regulatory-binding protein. The former is responsible for the regulation of water transport across brush border cells of the small bowel, whereas the latter has no specifically identified ligand, but this receptor class has been shown to be able to bind a wide range of ligands including protease inhibitors like α2-macroglobulin (down-regulation of inflammation), lipoproteins, extracellular matrix proteins, and growth factors/cytokines (33).

**Neuroendocrine peptides involved in regulation of food intake.** *vgf* is a secreted protein present in vesicles of neurons and peripheral neuroendocrine cells, especially in the adenohypophysis and the adrenal medulla. The pro-protein is processed by pro-hormone convertase (see *Genes implicated in neuroen-


docrine secretion), resulting in tissue-specific small secreted peptides. Recent studies showed a complex pattern of different vgf-derived peptides in neuroendocrine cells of the stomach (30). vgf is regulated in the hypothalamus in response to feeding (17) and in the dorsal vagal complex of the medulla oblongata in response to duodenal ulcer formation. This indicates a possible role in energy balance of the body via control of food ingestion (48). Interestingly, vgf expression is also regulated in the suprachiasmatic nucleus (SCN; master core circadian clock) in response to the light-dark cycle (18). ECL cells strongly and specifically express vgf, indicating a peripheral phase of secretion of this peptide in addition to central regulation, most likely in the local control of acid secretion and in a circadian response, perhaps accounting for nocturnal acid secretion that is sensitive to H2 receptor antagonists as discussed below.

Regulation of circadian rhythm. The stomach shows a secretory circadian rhythm resulting in diurnal oscillations of acid secretion (28), cell kinetics (13), and bicarbonate and mucous secretion (29). These oscillations that occur during fasting are not mediated by gastrin stimulation of acid secretion (35) and can contribute to physiological phenomena like the anticipation of the body to an upcoming meal [i.e., early morning acidification in relation to breakfast (28)]. Nocturnal acid secretion, since it is sensitive to H2 receptor antagonists (46), may also reflect a circadian rhythm oscillation of ECL function. These circadian oscillations may play a role, if disrupted, in the pathophysiology of acid-related disorders like ulcer disease (35) and gastrointestinal esophageal reflux disease (GERD) (57) and nocturnal acid breakthrough, NAB (47). It is therefore of interest that the ECL gene expression profile shows strong expression of Gprasp1 [G protein-coupled receptor (GPCR)-associated sorting protein], which is also known as period 1-interacting protein (PIPS). This protein binds both to the COOH terminus of GPCRs (51) and to period 1, which is an essential part of the core circadian oscillator (9). Period 1 is also strongly expressed in ECL cells (normalized intensity of 19,188 compared with 10,000 for Na-K-ATPase α1-subunit) but must be present in other cells of the gastric mucosa, since we found only a threefold higher expression in ECL cells compared with the genes of the total gastric fundic epithelium. The presence of these genes could indicate that ECL cells express a core circadian oscillator that could be regulated through GPCRs and result in circadian rhythm of histamine biosynthesis and release and hence acid secretion.

Apelin and APJ Receptor Expression and Function in the Rat Gastric Mucosa

Apelin and APJ receptor expression in ECL and PC. Control of acid secretion is essential for the proper function of the gastric mucosa. Uncontrolled acidification of the gastric lumen leads to development of disease, including erosive gastritis and ulceration, and to a loss of ions, including potassium, chloride and protons, perhaps with the development of metabolic alkalosis due to an increase in blood bicarbonate concentration [alkaline tide (37)]. Therefore, one can assume that negative-feedback mechanisms to downregulate acid secretion in PC after stimulation may exist. Inhibition can occur in the PC itself [i.e., high amounts of phosphodiesterase to downregulate the major stimulatory histamine pathway (36), rapid internalization of histamine-H2 receptor complexes (52), by the presence of inhibitory receptors on PC, i.e., somatostatin receptor SSTR2 (50)] and by a paracrine (somatostatin from D cells) or neural [galanin (69)] downregulation of histamine release in ECL cells. This downregulation suggests that neuroendocrine
cells or neurons sense a stimulus after successful acid secretion to release inhibitory peptides, a mechanism that is not well understood and that could be rather slow.

However, if PC were able to release an inhibitory paracrine peptide in response to histamine stimulation that directly inhibits histamine release in ECL cells locally, a much faster but so far unexpected negative-feedback regulation between the two cell types is possible. The PC and ECL cell gene expression profiles indeed show such a negative-feedback peptide-receptor pair: apelin in PC and the APJ receptor in ECL cells (Table 2). Apelin (APJ receptor endogenous ligand) is specifically expressed by PC [ratio = 2.54 (27)] but not ECL cells (ratio = 0.48) compared with total gastric fundic epithelium. This is a recently identified endogenous peptide present in bovine stomach extracts (65) that binds to the orphan receptor APJ (angiotensin-like receptor-1) (40). We found specific APJ receptor expression in ECL cells (ratio = 8.22) but not in PC (ratio = 0.66).

Immunolocalization of apelin in PC. Immunolocalization studies using rat gastric sections colabeled with a polyclonal antibody against apelin (Fig. 5A, red) and a monoclonal anti-HK-ATPase antibody (Fig. 5B, green) show colocalization of both antigens in PC (Fig. 5C, yellow composite color) as had been previously shown (62, 66). Higher magnification of PC (Fig. 5D) shows that apelin staining is identified in a vesicular pattern in the basal pole of PC facing the interstitium between the gastric glands.

**Immunolocalization of APJ receptor in ECL cells.** Immunolocalization of the APJ receptor in rabbit gastric glands showed neuroendocrine cells costaining for the receptor and HDC, a specific marker of ECL cells. High-resolution scans of the cells showed that the receptor is located peripherally in linear and vesicular patterns, consistent with membranous and endosomal localization, in contrast to HDC, which was mostly found in perinuclear vesicles (Fig. 6).

Apelin inhibition of gastrin-stimulated intracellular calcium signaling in ECL cells. The identification of apelin in PC and its receptor (APJ receptor) in ECL cells initiated an investigation of the functional effect of apelin on histamine- and gastrin-stimulated intracellular calcium concentrations in the well-established model of the isolated rabbit gastric gland. This model allows visualization of changes in intracellular calcium
concentration of all different cell types present in the glands using the fluorescent calcium ion-binding probe Fluo4/AM. Distant cell-to-cell cross talk due to neuropeptide release in the bath solution is avoided using rapid medium superfusion where the medium is exchanged four times per minute. If 1 nM gastrin I is used (Fig. 7A, tick mark 1), only cells that appeared to be mostly on the bottom of the glands and peripheral to the lumen responded with an initial spiking of the calcium signal (release from intracellular calcium stores) followed by a plateau phase (entry via receptor-operated calcium channels) as typically seen in ECL cells (cell 1). After a short delay, neighboring PC reacted with a broad calcium elevation devoid of the initial spike (entry via receptor-operated calcium channels only, cell 2), which at 1 nM gastrin concentration is due to histamine release from the ECL cell and its action on the nearby PC and is not due to a direct action of gastrin at this low concentration as previously reported in detail (3). The ECL cell response to 1 nM gastrin I was almost completely abolished in the presence of 100 nM apelin (time point 3). After the apelin is washed out (time point 4), rechallenge of the system with gastrin results in Ca2+ signal only in the ECL cell at 1 nM concentration (time point 5). To show PC viability at the end of the experiment, 10 nM gastrin was used (time point 6) resulting in a simultaneous increase in intracellular Ca2+ concentrations in both cells mediated by the high-affinity gastrin receptor on ECL cells and the low-affinity receptor on PC, as previously published (3).

Apelin inhibits gastrin-stimulated aminopyrine uptake in rabbit gastric glands. We tested the effect of apelin in a quantitative model of acid secretion using aminopyrine (AP) uptake in rabbit gastric glands (5). Figure 7B shows the AP ratio (difference of AP between extracellular and intracellular compartments) during various conditions of stimulation and inhibition of acid secretion. There is routinely ~40–50% AP uptake stimulation in gastrin I-stimulated glands compared with histamine-stimulated glands, as seen in previously published results (7). Both histamine- and gastrin-stimulated AP uptake was inhibited by the addition of 10 µM cimetidine. Apelin abolished the gastrin-stimulated AP uptake in this system but had no effect on histamine stimulation. Hence the action of apelin is on the ECL cell and not the PC. These data demonstrate a direct inhibitory effect of apelin on ECL cell release of histamine during stimulation of acid secretion in rabbit gastric glands.

In summary, a more detailed understanding of the biology of ECL cells has been difficult because of the relatively low percentage of this cell in the gastric fundic mucosa and the inability to purify this cell to homogeneity without short-term culture (42, 69). Isolation of >95% pure ECL cell suspensions and gene expression analysis thereof led to the description of at least six major functions of this important neuroendocrine cell, some of which were previously undescribed. These include (1) neuroendocrine peptide synthesis, processing, and secretion (chromogranin A and B); (2) synthesis and release of peptides to control local inflammation (thrombomodulin, vitronection, muringlobulin); (3) release of growth factors for epithelial regeneration (Reg1 and NOV); (4) release of neuroendocrine peptides involved in regulation of food intake (vgl); and (5) expression of transcription factors coupled to GPCRs to maintain a circadian rhythm oscillator (period 1, PIPS). In addition, the expression profile identified several inhibitory receptors expressed on ECL cells, which could be crucial for downregulation of histamine release and the resulting inhibition of the peripheral phase of acid secretion (nicotinic acid receptor, apelin receptor, and perhaps GABA-B receptor). One of these receptors, the apelin receptor, was studied in detail because of the presence of its ligand, apelin, in PC. The functional data and immunohistochemical localization suggest that apelin released from PC binds to the APJ receptor expressed in ECL cells, thereby inhibiting histamine release. This novel negative-feedback regulation might provide a local control of acid secretion in the gastric mucosa.

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