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Isolation, functional characterization, and transcriptome of *Mastomys* ileal enterochromaffin cells

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Kidd, M., I. M. Modlin, G. N. Eick, and M. C. Champaneria. Isolation, functional characterization, and transcriptome of *Mastomys* ileal enterochromaffin cells. *Am J Physiol Gastrointest Liver Physiol* 291: G778–G791, 2006. First published February 2, 2006; doi:10.1152/ajpgi.00552.2005.—Although the enterochromaffin (EC) cell is one of the primary neuroendocrine regulatory cells of the small intestine, the lack of a purified cell system has precluded characterization of the cell and limited precise physiological evaluation. We developed methodology to obtain a pure population of *Mastomys* ileal EC cells, evaluated their functional regulation, and defined the transcriptome. *Mastomys* ilea were everted, end ligated, pronase-collagenase digested, and Nycodenz gradient centrifuged, and EC cells were collected by fluorescence-activated cell sorting (FACS) of acridine orange-labeled cells. Enrichment was confirmed by immunostaining of tryptophan hydroxylase and chromogranin A, specific EC cell markers, serotonin content, EC cell marker gene expression, and electron microscopy. Pituitary adenylate cyclase-activating polypeptide (PACAP), somatostatin, and gastrin receptor expression was determined by real-time RT-PCR. Live post-FACS-sorted cells were cultured, and the effects of forskolin, isoproterenol, acetylcholine, $GABA_A$, PACAP-38, and gastrin on serotonin secretion were measured by ELISA. GeneChip Affymetrix profiling of FACS-sorted cells was undertaken to obtain the EC cell transcriptome. FACS produced a >70-fold enrichment of EC cells with a serotonin content of 240 ± 22 ng/mg protein. Preparations were 99 ± 0.7% pure by immunostaining for tryptophan hydroxylase. Vasoactive intestinal peptide/PACAP receptor 1 ($VPAC_1$) and somatostatin receptor 2 were present, whereas PACAP receptor 1 (PAC_1) and CCK_2 receptors were undetectable. Forskolin, isoproterenol, and PACAP-38 stimulated serotonin secretion at EC_{50} values of 5×10^{-10}, 4.5×10^{-10}, and 1.2×10^{-9} M, respectively. Isoproterenol stimulated cAMP levels by ~3.5 ± 0.62-fold vs. unstimulated cells (EC_{50} of $\sim 10^{-9}$ M). Octreotide, acetylcholine, and $GABA_A$ inhibited serotonin secretion with IC_{50} values of 3×10^{-11}, 3×10^{-10}, and 2.9×10^{-10} M, respectively. Gastrin had no effect on serotonin secretion. The naive EC cell transcriptome revealed highly expressed EC cell marker genes, the absence of marker genes for other small intestinal cell types, and a receptor profile that included cholinergic, adrenergic, dopaminergic, serotoninergic, GABAergic, and prostaglandin receptors. We were able to isolate homogeneous preparations (>99%) of live ileal EC cells and demonstrated regulation of serotonin secretion as well as established the normal EC cell transcriptome. Application of this methodology to normal and diseased human ileum will facilitate the elucidation of the pathophysiology of EC cells.

fluorescence-activated cell sorting; serotonin; secretion; small intestine; carcinoid

THE ENTEROCHROMAFFIN (EC) cell is distributed throughout the gastrointestinal epithelium and is regarded as the predominant neuroendocrine (NE) cell of the bowel (48). Its location within glands at the base of intestinal crypts and its basal extensions projecting into adjacent glands attest to its local regulatory role (9). Although generally thought of as a specific cell type, EC cells may comprise a number of different subpopulations (41); the majority of cells are of the "open" type with apical cytoplasmic projections covered with microvilli, but some appear to be of the "closed" endocrine phenotype with no discernible luminal access (49). Of the six endocrine cells described in the terminal ileum, the EC cell is by far the most frequently occurring (~40%) (44). Other endocrine cells identified in this location include enteroglucagon/peptide YY (PYY) (EG/L cells), neurotensin (N cells), somatostatin (SST; D cells), gastrin (CCK cells), and pro-γ-melanocyte-stimulating hormone (MSH) cells (48).

Serotonin [5-hydroxytryptamine (5-HT)] is synthesized in the ileal EC cell by a mechanism involving the EC cell-specific enzyme tryptophan hydroxylase (TPH) (52) and stored in secretory vesicles via a process mediated by vesicular monoamine transporter 1 ($VMAT_1$) and proton gradients. It is released by mechanisms that are not well defined in response to a number of stimuli including mechanical stimulation or acidification of the gut lumen (25, 52). Serotonin is released from the basolateral membrane of the ileal EC cell and exerts a paracrine effect on adjacent enterocytes and smooth muscle cells as well as passing into the circulation as a classic endocrine regulator (19). More recently, it has become apparent that in addition to serotonin, ileal EC cells synthesize and secrete substance P and guanylin (8, 44). Both of these agents have been identified as modulators of intestinal secretion and motility (8, 53), but little is known of the regulatory events associated with their release from the ileal EC cell. Nevertheless, the ileal EC cell, by virtue of producing at least three small intestinal regulatory agents, exhibits a pivotal role in the physiology of small intestinal function and most likely in its disease states such as irritable bowel syndrome and carcinoid tumors (11, 34, 53).

It is apparent that the ileal EC cell is one of the primary NE regulatory cells of the small bowel, and, given its wide distribution and large numbers, it may occupy as dominant a role in the small intestine as that of gastrin in the stomach (36). To date, however, the ileal EC cell has proved difficult to study given its scattered location amid a wide variety of different cell types. Serotonin release has been examined in a number of ex vivo preparations, and these studies have demonstrated that it is mediated via a number of agents including mucosal stimulation by fat and PYY (52) and cholinergic (acetylcholine) and adrenergic (e.g., norepinephrine) nerves (52). In addition, pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal polypeptide (VIP) appear to have potent

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inhibitory effects on serotonin release (16). The interpretation of such observations is restricted by the fact that intact mucosal preparations preclude, to a large extent, the determination of direct effects of specific agents on the ileal EC cell itself. Furthermore, little is known about the roles and interactions of the other five ileal NE cell types that may participate in modulating either mucosal or EC cells (52). Given the heterogeneous nature of the mucosa within which the ileal EC cell is distributed, precise characterization of the cell would be facilitated by developing a homogenous ileal EC cell preparation that can be used to identify its transcriptome and define its response to stimuli.

We and others have reported methodologies of varying effectiveness for the isolation and short-term culture of a number of different gastrointestinal endocrine cells from animal models including *Mastomys* (2, 6, 24, 26, 42). With the use of elutriation and Nycodenz gradient centrifugation of everted normal and carcinoid-containing stomachs, 85–95% pure preparation of gastric EC-like (ECL) cells can be obtained (26, 31). Studies of purified gastric ECL cells have demonstrated the key regulatory role of the histamine enzyme histidine decarboxylase in gastric ECL cell histamine secretion, identified the presence of gastrin CCK_2 and PACAP receptors and defined their key roles in proliferation and secretion (28, 31, 32, 50), and delineated the presence of SST receptor 2 (SST_R2) and the inhibitory role of octreotide in both secretion and proliferation (4, 45). With the use of a somewhat similar approach, preparations of neurotensin and enteroglucagon cells have been obtained from the canine ileum (2, 6). Assessment of these cells demonstrated a key role of cAMP activation by forskolin and β-adrenergic receptor activation by isoproterenol in the secretion of neurotensin and enteroglucagon (2). More recently, Schafermeyer et al. (46), using counterflow elutriation and step density gradient centrifugation, have reported preparations of rat ileal EC cells with 15–20% contamination. Preparations of these cells were used for both in vitro serotonin release experiments and transcriptional profiling. The presence of cell contamination with other NE cells (47), as well as the high chemical doses (10^{-5}–10^{-4} M) required to elicit serotonin release in these cells (46), suggests the need to develop homogenous ileal EC cell preparations.

More recently, a fluorescence-activated cell sorting (FACS) approach has been utilized to obtain homogeneous (~99% pure) parietal and ECL cell populations from the rat gastric mucosa (29, 38). This methodology has not been applied to ileal cell preparations. In the present study, we outline the application of intestinal eversion, digestion, Nycodenz gradient centrifugation, and FACS using anti-TPH and acridine orange (AO) labeling to derive a pure population of *Mastomys* ileal EC cells and define their transcriptome and functional regulation in vitro.

METHODS

Our primary goal was to isolate, enrich to homogeneity, and characterize the function of EC cells isolated from *Mastomys* ilea. EC cells were collected by FACS of TPH-positive or AO-positive fractions collected after Nycodenz gradient centrifugation. TPH is an EC cell-specific enzyme that catalyzes serotonin formation, whereas AO is a weak base that accumulates in NE cell vesicles due to their low intravesicular pHs (15), a property that has previously been used to visualize NE cells (42). Enrichment of EC cell preparations was evaluated by immunostaining, serotonin content, and marker gene expression (PCR). The transcripts of candidate regulatory receptors were confirmed by real-time RT-PCR. The viability and secretory responses of live cells were then determined in vitro.

Mastomys Tissue Specimens

We developed the methodology using ileal tissue from the multimammate mouse (*Mastomys natalensis*). Although our eventual goal is to define the human EC cell phenotype, we used this model because we have extensive experience in isolating and characterizing the gastric equivalent of the EC cell—the ECL cell—from this animal (26, 50), and the close phylogenetic relationship of *Mastomys* with the mouse allowed the use of murine Affymetrix GeneChip for transcriptome analysis of *Mastomys* EC cells (26). *Mastomys* with equal sex distribution were used. *Mastomys* (n = 4 animals/preparation) were euthanized by CO_2 inhalation and cervical dislocation. We used the terminal 15 cm of the *Mastomys* small intestine (total length = 48 ± 4.3 cm, duodenum to cecum) as representative of the ileum (40). This was removed and divided into two to three pieces, which were everted and end ligated. All animal studies were approved by Yale University (IACUC no. 2004-10904).

Cell isolation. Everted, end-ligated ilea were filled with pronase E (0.7 mg/ml) and collagenase (0.25 mg/ml) and preincubated in DTT-HBBS for 30 min to remove mucus (6, 46). Cells were then separated from the muscularis by alternative switching between a calcium-containing respiration medium and a chelating digestive medium (with stirring) (the F_0 fraction; Table 1). Ileal mucosal (IM) scrapings were retained to provide a baseline to assess enrichment.

Nycodenz gradient centrifugation Density gradient centrifugation at 1,100 rpm was performed with the F_0 faction, and cells were collected at 1.070 g/l (the F_N fraction) (46).

Preparation for FACS. TPH-POSITIVE CELLS. Cells from the F_0 and F_N fractions were fixed in ice-cold methanol (−20°C, 100%, final concentration = 50% solution) for 5 min, washed three times with PBS, and blocked with 0.1% BSA before being immunostained with mouse monoclonal anti-TPH antibody (0.1 μg/ml, Calbiochem, overnight at 4°C). Cells were then washed and stained for 20 min (at room temperature) with FITC-labeled secondary antibody (1:100).

AO-POSITIVE CELLS. Live cells from the F_N fraction were incubated with AO (2–200 μM) for 30 min (at room temperature) before being sorted.

FACS. All fractions were filtered (50-μm pore size) before being sorted. FACS analysis (high-speed FACS Aria, Yale Cancer Center) was used to identify and sort cells. Excitation was at 488 nm

Table 1. *EC cell purification from Mastomys ileum*

			F_{FACS}	
	F_0	F_N	TPH	AO
Serotonin compared with IM, fold difference	5±2.1	29±3.4	70±5.8	69±4.7
TPH-positive cells, %	2.7±1.2	80±3.6	98±1.2	99±0.7
CgA-positive cells, %	3.3±0.7	81±5.6	100±1.1	100±0.6
Cell number	1.3×10^7	4.8×10^6	6.6×10^5	7.3×10^5
Viability, %	99.3	97.2	N/A	94

Values are means ± SE; n = 5 *Mastomys* enterochromaffin (EC) cell preparations. Cell numbers are the averages of 5 separate experiments (4 animals/experiment). F_0, initial mucosal preparation; F_N, F_0 preparation after Nycodenz gradient centrigugation; F_{FACS}, F_N preparation after fluorescence-activated cell sorting (FACS) with either tryptophan hydroxylase (TPH) or acridine orange (AO). Nycodenz gradient centrifugation followed by FACS with either TPH- or AO-stained cells results in sequential enrichment of ileal EC cells. IM, intact mucosa; CgA, chromogranin A. Viability was assessed by Trypan blue exclusion. TPH-F_{FACS} fraction viability was not evaluated because cells were ethanol fixed before anti-TPH immunostaining [not applicable (N/A)].

(activation of FITC- or AO-labeled cells), and sorting was achieved by gating on side scatter (dense, small cells of an estimated ~8–12 μm size) and an emission of 532 ± 15 nm. We collected positive cells over 30 min (the F_{FACS} fraction). To demonstrate that AO uptake was due to the generation of acidic spaces within granules, aliquots (n = 3) were preincubated with 20 mM NH_4Cl for 30 min before the AO addition and FACS.

Immunostaining. TPH-POSITIVE CELLS. After FACS, an aliquot of FITC-TPH-positive cells (20–50,000 cells) were pipetted onto frosted microscope slides. Fluorescence microscopy was used to visualize and count the number of positive cells to give the percentage of TPH-positive cells after FACS. A second aliquot of these FACS-sorted cells were incubated with chromogranin A (CgA) antibodies (1:200; goat, Santa Cruz Biotechnology) overnight at 4°C. After being washed (0.1% Tween-PBS), cells were stained with secondary antibody (horseradish peroxidase-conjugated anti-goat, 1:100, DAKO) for 1 h at room temperature and visualized with a fluorescent chromogen (Cy5-tyramide, NEN Life Science Products) to give the percentage of CgA-positive cells (NE cells in each preparation).

AO-POSITIVE CELLS. Live AO-FACS-sorted (50,000 cells/antibody) EC cells were fixed in methanol and pipetted onto frosted microscope slides. Preparations were stained with anti-CgA (mouse, 1:1,000, DAKO Cytomation), anti-TPH (mouse, 0.1 μg/ml, Calbiochem), or anti-serotonin (mouse, 1:20, DAKO Cytomation) antibodies overnight at 4°C. After being washed (0.1% Tween-PBS), cells were stained with secondary antibody (FITC-conjugated Cy5-anti mouse/rabbit, 1:100, Promega) for 1 h at room temperature. Cells were visualized and counted to assess the percentage of NE or EC cells in each preparation. As a control for nonspecific staining, primary antibodies were excluded. For confocal microscopy, micrographs were recorded using a confocal microscopy system equipped with three Ar488, Kr568, and HeNe633 lasers (TCS-SP, Leica; Mannheim, Germany) with a ×40 objective.

EC CELLS IN SMALL INTESTINAL MUCOSA. Small intestinal mucosa were obtained after the animals were euthanized, washed in PBS, and immersed in 4% formaldehyde overnight (28). Fixed tissue was mounted edge-on in paraffin blocks. Deparaffinized sections were studied using hematoxylin and eosin staining for the assessment of EC cell numbers by immunostaining with TPH (as above). The numbers of TPH-positive cells were assessed by examining sections (magnification: ×450) using a square grid of 10 × 10 mm (American Optical; Buffalo, NY) and noted as the numbers of cells per 1-mm^2 zone. Only the areas with full-thickness mucosa were measured. An average of 10 grids from each section were counted. The cell density of TPH-positive cells was calculated as follows (expressed as cell numbers/mm^2):

$$\text{Density} = (\text{total number of positive cells in all grids counted}/\text{number of grids}) \times (450/100\ \text{mm}^2).$$

Electron microscopy. Cells were evaluated using the semithin technique (39). Isolated cells (500,000 cells) were pelleted and fixed with 3% gluteraldehyde in 0.1 M cacodylate buffer (pH 7.4). After osmification, cells were dehydrated with graded alcohol and embedded in Epon. Sections were evaluated with a Philips 300 transmission electron microscope. EC cells were distinguished from other NE cells by the presence of pleomorphic granules (oblong, ovoid, kidney shaped, triangular, or U shaped) that can be identified ultrastructurally (7).

PCR. Three markers of EC cells were assessed using real-time PCR (CgA, $VMAT_1$, and TPH). For gastrin, PACAP, SST, and GABA receptor expression, CCK_2 receptor, PACAP receptor 1 (PAC_1) and VIP/PACAP receptor 1 ($VPAC_1$), SST_R2, and $GABA_A$ and $GABA_B$ receptors (respectively) were assessed using a real-time approach (26). All primer probe sets were inventoried Assay-On-Demand probe sets obtained from Applied Biosystems. To detect contaminating NE cells, neurotensin, SST, glucagon, and PYY transcripts were amplified by RT-PCR.

RNA EXTRACTION AND CLEANUP. RNA from the various fractions (IM, F_0, F_N, and F_{FACS}) was extracted using TRIzol (Invitrogen), and then cleaned up using the Qiagen RNeasy Kit in conjunction with the DNeasy Tissue Kit (Qiagen) to minimize contaminating genomic DNA. The cleaned-up RNA (2 μg) was converted to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems).

REAL-TIME PCR PROTOCOL. Real-time RT-PCR analysis was performed using Assay-On-Demand products according to the manufacturer's suggestions. Cycling and fluorescence detection were performed using the ABI 7900 Sequence Detection System. Only Assay-On-Demand primers designed to hybridize to exon:exon boundaries (TaqMan Gene Expression Assays Product Guide) were used, thereby minimizing the possibility of amplifying genomic DNA. Controls without reverse transcriptase were included in triplicate in each real-time RT-PCR experiment to ensure the absence of genomic DNA contamination. Cycling was performed under standard conditions as outlined in the TaqMan Universal PCR Master Mix Protocol, and the Standard Curve Method (ABI User Bulletin No. 2) was used to determine relative transcript levels. *GAPDH* was used to normalize data.

RT-PCR. PCRs were performed in a 25-μl volume containing 0.2 mM of each dNTP, 2.0 mM of 1× reaction buffer (Roche PCR core kit), 0.2 M of forward and reverse primers, 0.5 units of *Taq* DNA polymerase (Roche), and 100 ng of template DNA. Thermal cycles consisted of 35 cycles of 1 min at 94°C, 1 min at annealing temperatures (50–55°C), and 1 min at 72°C, followed by an extension for 10 min at 72°C. Amplification of *GAPDH* transcript (5) in all samples was used as a positive control. Samples were loaded on 4.5% Supra Sieve agarose gels (American Bioanalytical) and visualized by ethidium bromide incorporation (0.5 μg/ml) under ultraviolet light.

Serotonin content. Serotonin content was measured and quantified as a percentage of the total protein content. Briefly, 50,000 cells were pelleted by centrifugation at 300 *g* for 5 min. Total protein was extracted by homogenization of the cells in lysis buffer [50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaF, 10 mM $Na_4O_7P_2$, 1 mM Na_3VO_4, 10 mg/ml leupeptide, and 1 mM PMSF]. The concentration of protein (in mg/ml) was assayed using the Bio-Rad Protein Assay. Serotonin content (in ng/ml) of each fraction was then measured using a commercially available serotonin ELISA (Diagnostika) according to the manufacturer's instructions for serum samples. Serotonin content was defined as nanograms of serotonin per milligram of protein.

Culture, serotonin secretion, and cAMP experiments. Live AO-FACS-sorted cells were divided into aliquots (~3×10^4 cells/100 μl), placed in culture media [serum-free Ham's F-12 medium (GIBCO-BRL) supplemented with antibiotics (100 units penicillin/ml + 100 μg streptomycin/ml, Sigma-Aldrich)], plated into 96-well collagen I-coated plates (Becton Dickinson), and maintained in a humidified atmosphere at 37°C in 5% CO_2.

After 2 h, a time course for serotonin secretion in response to the β-adrenergic receptor agonist isoproterenol (10^{-6} M) was evaluated. The latter agent was chosen based on its reported agonist effect on ileal neurotensin- and enteroglucagon-secreting cells (2, 6). Cells were stimulated with isoproterenol or media alone (control) for 0, 3, 5, 15, 30, 60, 90, and 120 min. To confirm that secretion represented a secretory process rather than cell damage or lysis, lactate dehydrogenase (LDH) release into media was measured using a commercially available LDH assay (CytoTox-ONE Homogenous Membrane Integrity Assay, Promega) according to the manufacturer's instructions for cultured cells.

Having determined the optimal time course for secretion, the effects of isoproterenol on intracellular cAMP were measured using a commercially available ELISA kit (R&D Research). Briefly, ~5×10^4 cells/100 μl in culture media were stimulated with isoproterenol (10^{-10}–10^{-6} M) for 15 min, and cAMP was assayed and expressed as picomoles per milliliter.

Individual serotonin secretion dose-response curves for forskolin, isoproterenol, acetylcholine chloride, $GABA_A$, PACAP-38, and gastrin (all 10^{-12}–10^{-6} M) were then established. The cAMP activator forskolin was utilized because it has previously been used as a positive control for ileal neurotensin and enteroglucagon cell secretion (2, 6). To determine whether naive EC cell secretion was subject to influence by SST_R activation, the effects of octreotide (10^{-12}–10^{-6} M) on isoproterenol ($EC_{50} = \sim 5 \times 10^{-10}$ M) were also evaluated. Media were collected from each well after 15 min of stimulation, and serotonin secretion was measured using a commercially available serotonin ELISA (Diagnostika). Serum-free growth medium was used as a blank solution. The data for serotonin secretion were expressed as percentage changes from baseline secretion, and values are means ± SE for each concentration. LDH secretion was measured in parallel experiments to verify that EC cell serotonin secretion did not represent a damage-related release. The viability of cells was also assessed using Trypan blue exclusion. Equal volumes of Trypan blue and the cell suspension were mixed and, after 5 min, loaded on a Brightline hemocytometer. Cells (1×10^3) were visualized, counted using light microscopy (magnification: ×40), and expressed as percentages of viable cells.

GeneChip examination. To obtain the naive EC cell transcriptome, we utilized Affymetrix profiling of AO-FACS-sorted preparations.

GENECHIP HYBRIDIZATION. For each experiment ($n = 3$), 2–3.5 μg of high-quality (ratio >1.9) total RNA isolated from AO-FACS-sorted cells or RNA isolated from the IM (whole mucosal scraping from the terminal 15 cm of the ileum) were provided to the Keck Affymetrix facility to perform cRNA labeling, hybridization, and data analysis. A standard protocol (Keck Affymetrix Core Facility, Yale) was used before hybridization to the Mouse Expression 430 2.0 chip (39,000 transcripts).

GENECHIP DATA ANALYSIS. The hybridized arrays were scanned using an Affymetrix GeneChip 3000 Scanner. Arrays were scaled to an average intensity of 500, and hybridization intensity data were converted into presence/absence calls for each gene using GCOS software. The signal intensity data were statistically analyzed using S-plus 2000 software (Mathsoft; Cambridge, MA) to obtain correlation coefficients and scatter plots for the evaluation of the reproducibility and quality of the array analysis. We used both the normalized array intensities and present calls to identify the full complement of transcripts in these preparations. This pattern of genes was used to define the EC cell transcriptome. In a separate analysis, a Wilcoxan rank test was used to compare expression levels in the EC cell preparations with those in the IM. Genes with relatively high expression (>2-fold) were identified and catalogued for the EC cell.

Statistical analyses. Results are expressed as means ± SE; *n* indicates the numbers of preparations. Data are represented as the fold differences vs. basal values. Statistical significance was calculated by the two-tailed Student's test for paired and unpaired values as appropriate, with a probability of <0.05 representing significance.

RESULTS

EC Cell Enrichment

Nycodenz gradient centrifugation. In the intact IM, the density of TPH-positive EC cells was 0.6 ± 0.23% by immunostaining. The serotonin content of the mucosa was 3.5 ± 0.6 ng serotonin/mg total protein. After eversion and pronase-collagenase digestion, the F_0 fraction contained 2.7 ± 1.2% TPH-positive cells and 18 ± 0.5 ng serotonin/mg total protein (Table 1 and Fig. 1*A*). The process of digestion (development of a crude mucosal fraction) therefore provides an "apparent" enrichment for serotonin-containing cells. The most likely explanation is via loss of enterocytes with a subsequent "apparent increase" in EC cells. Nycodenz gradient centrifugation produced a 33-fold enrichment of serotonin-containing EC cells above that in the F_0 fraction. The F_N fraction had a serotonin content of 102 ± 12 ng/mg protein. For both the F_0 and F_N fractions, >99% and 97% of cells, respectively, were viable as assessed by Trypan blue exclusion.

FACS. In preliminary studies, FACS of methanol-fixed TPH-stained preparations from the F_0 and F_N fractions resulted in EC cell enrichments of 88% and 98%, respectively, as assessed by TPH-positive quantitation using fluorescent microscopic visualization. We therefore used FACS of the F_N fraction as the preferred method for obtaining homogenous EC cell

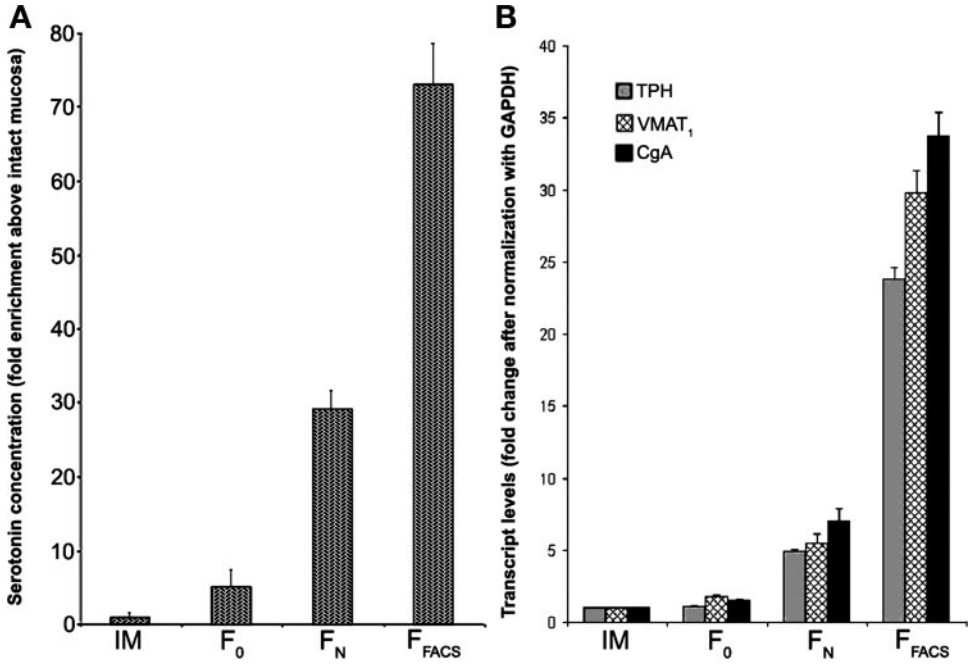

Fig. 1. Enrichment of *Mastomys* ileal serotonin-containing cells by Nycodenz gradient centrifugation and fluorescence-activating cell sorting (FACS). *A*: Nycodenz gradient centrifugation (F_N fraction: 102 ± 12 ng) and FACS (F_{FACS} fraction: 240 ± 20 ng) significantly enriched serotonin content [enterochromaffin (EC)-containing cells] from the crude cell fraction (F_0 fraction: 18 ± 0.5 ng). The lowest serotonin content was noted in the intact ileal mucosa (IM: 3.5 ± 0.6 ng). Values are means ± SE; $n = 3$. *B*: increase in transcript levels of tryptophan hydroxylase (*TPH*), vesicular monoamine transporter 1 (*VMAT1*) and chromogranin A (*CgA*) over the IM (fold increase) during progressive purification of EC cell fractions. Values are means ± SE; $n = 3$.

preparations. Fixation of the F_N fraction and staining of the preparations with anti-TPH antibody resulted in preparations of 98 ± 1.2% EC cells. Serotonin ELISAs confirmed enrichment, with serotonin levels increasing to >70-fold elevation over the whole mucosa in the F_{FACS} fraction (Fig. 1*A*). The serotonin content of 98% pure EC cells was 240 ± 22 ng/mg protein.

To produce live EC cells, we evaluated two different methodologies, fluoro-PACAP receptor binding and AO uptake. The fluoro-PACAP-labeling strategy has been previously utilized to isolate gastric ECL cells (38). Using the same conditions [fluoro-PACAP (0.1 μM); 30 min of pre-FACS incubation], we were unable to identify PACAP-positive *Mastomys* ileal EC cells (Fig. 2*C*). However, by increasing the labeling concentration of fluoro-PACAP to 10 μM, a discrete EC cell population could be identified. We considered that the ability to collect cells using an increased fluoro-PACAP concentration might reflect different levels of expression of the PACAP receptor subtypes on the EC cell compared with ECL cells and subsequently confirmed this by RT-PCR (21). Nevertheless, we considered fluoro-PACAP labeling (although successful; produced 96% pure EC cells) a suboptimal sorting strategy because it induces ECL cell function (secretion and proliferation) (38). Thus a fluoro-PACAP-sorted cell population would represent a prestimulated EC cell cohort and limit the ability to establish "true" baseline (nonstimulated) parameters of EC cell function.

We therefore adopted an alternative approach based on the ability of the fluorescent lysosomotropic weak base AO (used to visualize NE cells) to accumulate in the acidic vesicles of EC cells (15, 42). Thus accumulation in EC cell NE vesicles results in a fluorescent metachromatic shift from green (480 nm) to red (520 nm) (42). AO (2, 20, and 200 μM) staining of the F_N fraction (Fig. 2*D*) confirmed 99 ± 0.7% pure NE cells after FACS, and the additional use of TPH-positive immunostaining further established these FACS-sorted cells to be EC cells (Table 1 and Fig. 3*B*). Preincubation of cells with NH_4Cl before the addition of AO completely reversed the fluorescence, demonstrating the specificity of uptake. The serotonin content of these fractions was 69-fold increased relative to the IM.

Characterization of EC Cell Enrichment by Real-Time RT-PCR

To further evaluate and confirm sequential increase of the EC component of fractions, we performed real time RT-PCR to detect and quantitate EC-specific (*TPH* and *VMAT1*) and NE-specific (*CgA*) transcripts (Fig. 1*B*). Both NE transcripts as well as the EC-specific transcript *TPH* were sequentially enriched, with the F_{FACS} fraction (98–100% EC cells) showing the highest transcript expression for all markers (~24- to 34-fold vs. the IM).

Immunohistochemical and Ultrastructural Characterization of Homogenous EC Cell Fractions

The ultrastructure of F_{FACS} cells was evaluated using electron microscopy. Specific characteristics of EC cells (7, 49)

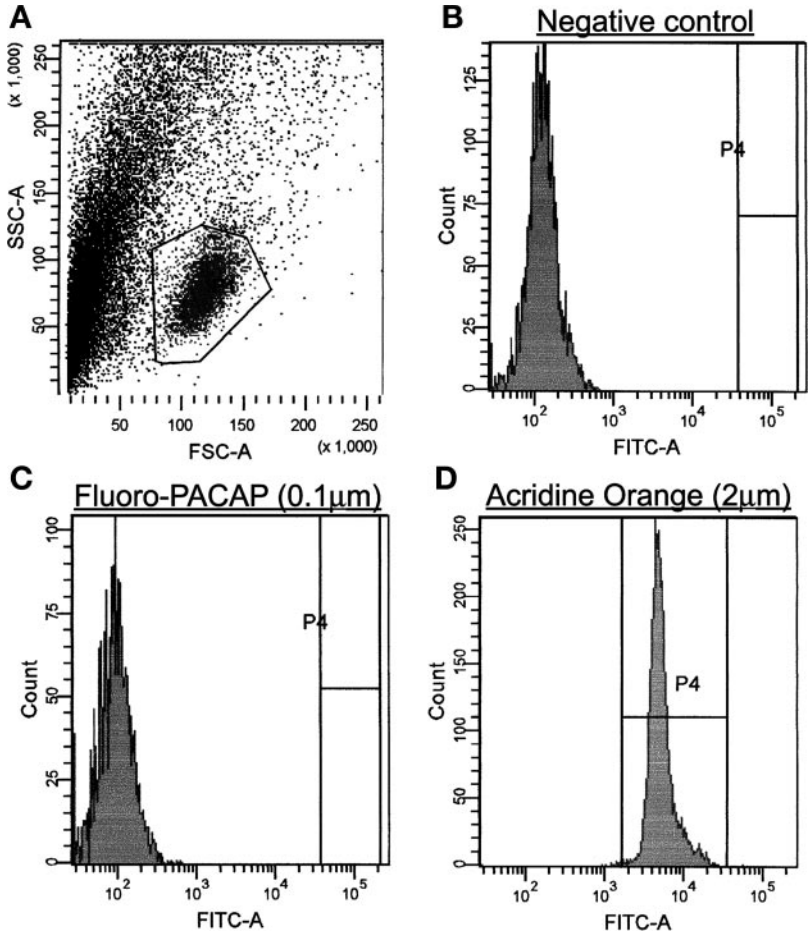

Fig. 2. FACS of live EC cells from the *Mastomys* F_N fraction. *A*: forward scatter (FSC)/side scatter (SSC) dot plot of unstained (TPH negative) and TPH-positive fractions. FSC provides a relative measure of cell size, whereas SSC provides a relative measure of cell density. A gate (*P1*) was defined around the major cell population to exclude high-density, large-sized cells and debris. *B–D*: histograms showing the shift to the right in FITC-labeled cells after immunostaining of live EC cells with FITC-labeled anti-IgG (negative control; *B*), fluoro-pituitary adenylate cyclase-activating polypeptide (PACAP; 0.1 μM; *C*), and acridine orange (AO; 2 μM; *D*). *Marker P4* was set to collected positive cells. Doublet and debris discrimination were performed before cells were sorted using standard procedures (data not shown).

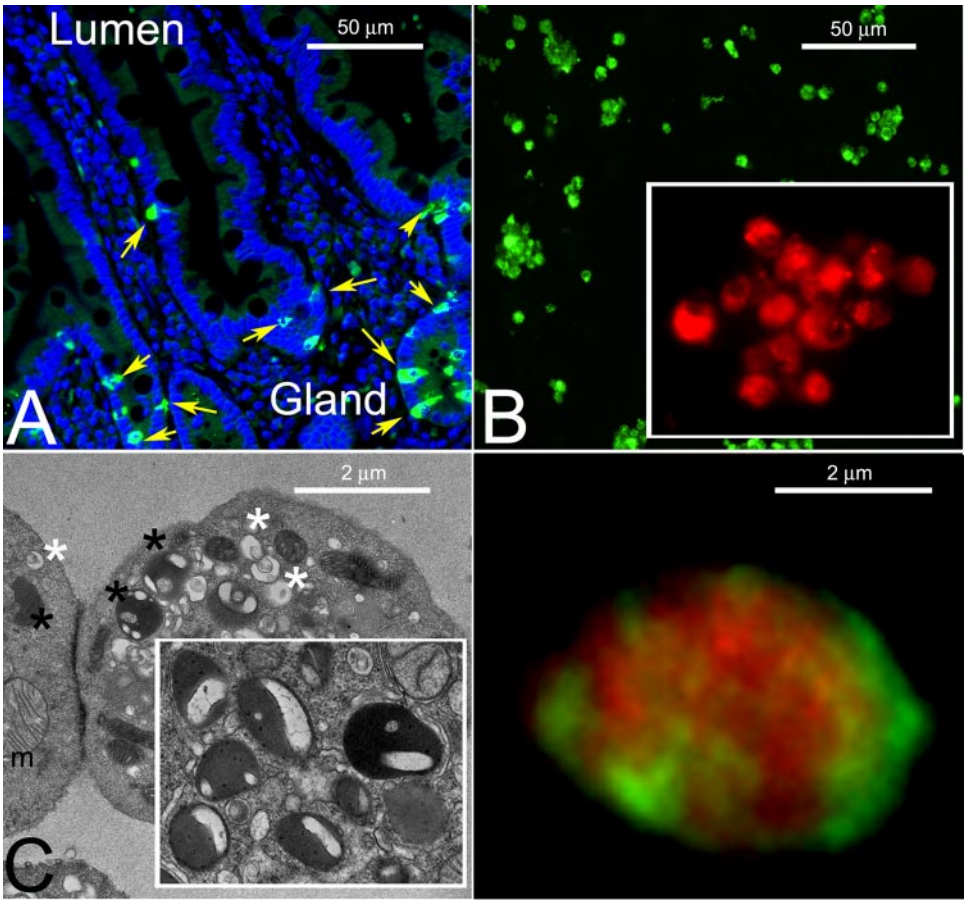

Fig. 3. EC cell topography. *A*: immunostaining of ileal EC cells (stained with FITC-conjugated anti-TPH antibody, yellow arrows) in glands within normal IM. Magnification: ×100. *B*: isolated ileal EC cells (FITC-conjugated anti-TPH antibody) from FACS and serotonin-positive cells demonstrating uniform staining. Magnification: ×100 and ×400 (*inset*). *C*: electron microscopy of EC cells demonstrating the typical admixture of large granules (black asterisks) and electroluscent empty vesicles (white asterisks). Vesicles are characterized by dense content and pear or ovoid shapes consistent with their proposed biconcave shape. m, Mitochondrion. Magnification: ×4,800 and ×9,600 (*inset*). *D*: confocal immunofluorescence microscopic picture of dual-stained EC cells demonstrating AO (green) and TPH (red) immunofluorescence within the cytoplasm. Colocalized staining is yellow. Original magnification: ×600, resized for the present image. Scale bars are shown.

could be confirmed, including the presence of *1*) well-developed Golgi apparati, numerous mitochondria, and rough endoplasmic reticula; *2*) vacuolated granules consistent with secretory function; and *3*) large numbers of variously sized electron-dense vesicles consistent with NE secretory function (Fig. 3*C*). Confocal microscopy of F_{FACS} cells dual stained with AO and TPH antibodies demonstrated colocalization of TPH and AO within the cytoplasm of these cells (Fig. 3*D*).

Identification of Contaminating Cells in the F_{FACS} Fraction

To identify the cell type of the 1% contaminating cells in the live cell preparations, we initially used monoclonal CgA (DAKO, 1:1,000) staining and demonstrated that 100% of cells in this FACS-sorted fraction were CgA positive (Table 1). This indicated the 1% contamination to be of NE cell origin. The enrichment techniques employed principally used size/density indexes and would be predicted to preferentially enrich NE cells, which are similar in size and density (7, 49). Although contamination represented only a very small percentage (<1%), we sought to identify the nature of these NE cells. According to intact mucosal immunohistochemistry, we considered the possibilities to include EG/L, N, D, CCK, or pro-γ-MSH cells (48). Accordingly, we utilized PCR to identify the specific contaminant NE cell types. In contrast to the F_0 fraction and IM, AO-FACS-sorted fractions did not contain SST, glucagon, gastrin, or PYY transcripts (Table 2). This fraction did, however, contain the neurotensin transcript, suggesting that the contaminating cell type was a neurotensin-producing cell. We further confirmed the absence of D cells by immunostaining the F_{FACS} fraction with an anti-SST antibody (1:2,000, ICN Biomedicals). In the F_N fraction, 4.3% of cells were SST positive, whereas SST levels were undetectable in the F_{FACS} fraction (Fig. 4, *A* and *B*). The absence of D cells in the 99% EC preparations is an important criterion because it ensures no extraneous inhibitory effect in the assessment of the proliferative/secretory profile of the EC cell (46).

PCR Characterization of Receptors

To identify receptors that might be responsible for the modulation of normal EC cell function, we used real-time PCR to examine acetylcholine, GABA, gastrin, PACAP, and SST receptors. A comparison of the IM with the F_{FACS} fraction

Table 2. *PCR-identifiable contaminant cells in Mastomys EC cell preparations*

	IM	F_0	F_N	AO-F_{FACS}
Somatostatin	+	+	+	−
Glucagon	+	+	+	−
Peptide YY	+	+	+	−
Gastrin	+	+	+	−
Neurotensin	+	+	+	+

The IM, F_0, and F_N preparations showed evidence of five neuroendocrine (NE) cell types, in contrast to AO-F_{FACS}-sorted cells, which expressed transcripts for one contaminating cell type: the neurotensin cell. +, Transcript present; −, transcript absent. *GAPDH* was detected in all samples.

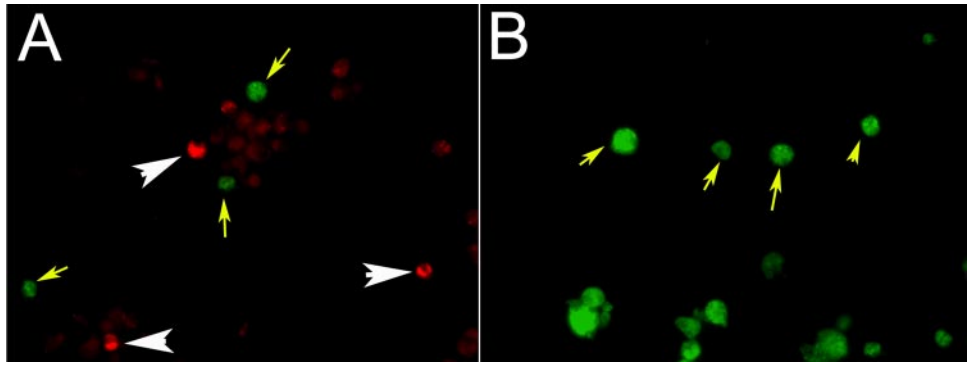

Fig. 4. Confirmation that somatostatin (SST)-containing cells are not present in the AO-FACS-sorted fraction. *A*: immunostaining the F_0 fraction with anti-TPH (green, FITC) and anti-SST (red, Cy5) antibodies demonstrated occasional TPH-positive and SST-positive cells in this preparation. Magnification: ×400. *B*: immunostaining of cells with FITC-labeled anti-TPH (green) antibody demonstrated 99–100% positive EC cells. None of the cells were Cy5-anti-SST positive. Magnification: ×400.

demonstrated transcripts for acetylcholine M_4, GABA-α_2, GABA-β_1, $VPAC_1$, and SST_R2 in both mucosal samples and FACS-sorted preparations (Fig. 5). PAC_1 and CCK_2 could not be detected in the latter preparations; results consistent with the EC cell transcriptome.

Functional (Serotonin Secretory and cAMP) Studies

Viability (assessed by Trypan blue exclusion) after the addition of 2, 20, or 200 μM AO and FACS was >94%, 67%, and 57%, respectively. Similarly, LDH secretion using the CytoTox-ONE Homogenous Membrane Integrity Assay was significantly elevated in the 20 and 200 μM AO-FACS-sorted fractions [595-nm optical density (corrected for blank) = 0.494 ± 0.032 and 0.651 ± 0.102] compared with the 2 μM AO-FACS-sorted fraction [595-nm optical density (corrected for blank) = 0.278 ± 0.018, $P < 0.01$, >70% elevation in LDH secretion].

Given the low viability of the 200 μM fraction, this preparation was not examined further, and the lower AO concentration was utilized in subsequent studies. Basal (unstimulated) serotonin release from the F_N fraction and 2 and 20 μM AO-FACS-sorted cells assessed in short-term culture (2 h) was 1.09, 1.04, and 0.26 $ng^{-1} \cdot 50{,}000\ cells^{-1} \cdot h$, respectively. The decrease in serotonin release in the 20 μM fraction presumably reflects vesicular damage caused by the high dose of AO and was confirmed by the associated high levels of LDH. On the basis of these measurements (unstimulated serotonin and LDH release), this fraction was therefore not further evaluated.

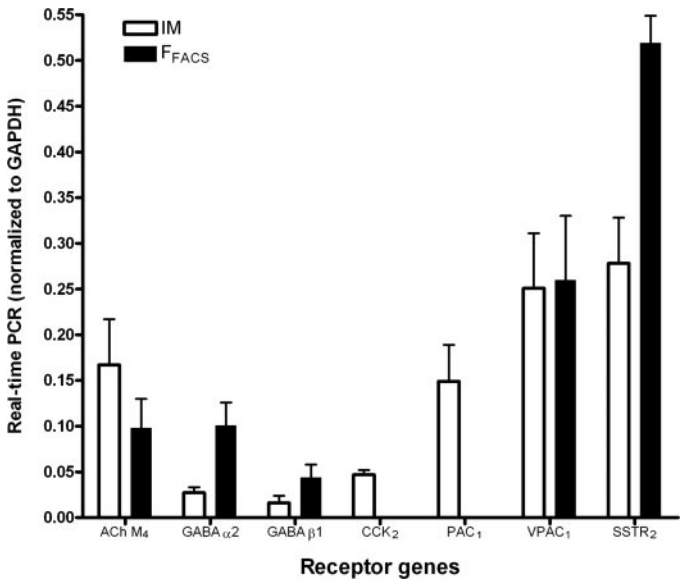

Fig. 5. Real-time PCR results of transcript levels in the IM or F_{FACS} fraction of the acetylcholine (ACh) M_4 receptor, GABA-α_2 and -β_1 receptors, gastrin (CCK_2) receptor, PACAP receptors PAC_1 and $VPAC_1$, and SST receptor SST_R2. Transcript levels are normalized to GAPDH. Expression of CCK_2 and PAC_1 mRNA was undetectable in F_{FACS} preparations. Values are means ± SE; $n = 3$.

Serotonin responses were assessed in AO (2 μM)-treated EC cells to isoproterenol (10^{-6} M) to establish the time course of secretion. Significant serotonin secretion was noted as early as 3 min in the media, reached a peak (55 ± 5%) at 15 min, and was detectable up to 120 min (>20% vs. media-stimulated cells) (all evaluated time points, $P < 0.05$ vs. unstimulated cells; Fig. 6*A*). An examination of LDH levels over this time period (120 min) demonstrated no effect of isoproterenol (Fig. 6*B*).

Isoproterenol has been reported to stimulate ileal neurotensin and enteroglucagon cell secretion by a mechanism that is considered to be cAMP mediated (2, 6). To examine whether EC cell serotonin secretion was also cAMP mediated, we measured intracellular cAMP levels in response to increasing concentrations of the β-adrenergic receptor ligand. At 15 min, isoproterenol stimulated cAMP levels by ~3.5 ± 0.62-fold vs. unstimulated cells, with an EC_{50} of $\sim 10^{-9}$ M (Fig. 6*C*). This effect was reversed by preincubating the cells for 15 min with 2′,5′-dideoxyadenosine (1 μM), an adenylate cyclase inhibitor (51). This demonstrates that the cAMP pathway is activated by this adrenergic receptor ligand in the EC cell. To develop a profile for the regulation of EC cell serotonin secretion, we initially assessed a number of agents previously reported to be effective in intact IM or heterogeneous cell preparations (gastric and ileal) containing NE cells (45, 55). Dose responses (at 15 min) were developed for secretagogues: the neural agents, isoproterenol, PACAP, acetylcholine chloride, and $GABA_A$, and the hormonal agents, gastrin and the SST agonist octreotide. Forskolin was also examined to assess the contribution of direct adenylyl cyclase activation in the stimulation of EC cell serotonin secretion.

Forskolin stimulated EC cell serotonin secretion with an EC_{50} of $\sim 5 \times 10^{-10}$ M, confirming cAMP signaling pathway involvement in serotonin secretion (Fig. 7*A*). Isoproterenol stimulated EC cell serotonin secretion with an EC_{50} of $\sim 4.5 \times 10^{-10}$ M (Fig. 7*B*). The SST_R2 ligand octreotide inhibited basal serotonin secretion by ~30% ($IC_{50} = 3 \times 10^{-11}$ M; Fig. 7*C*). Isoproterenol (EC_{50})-stimulated serotonin secretion was

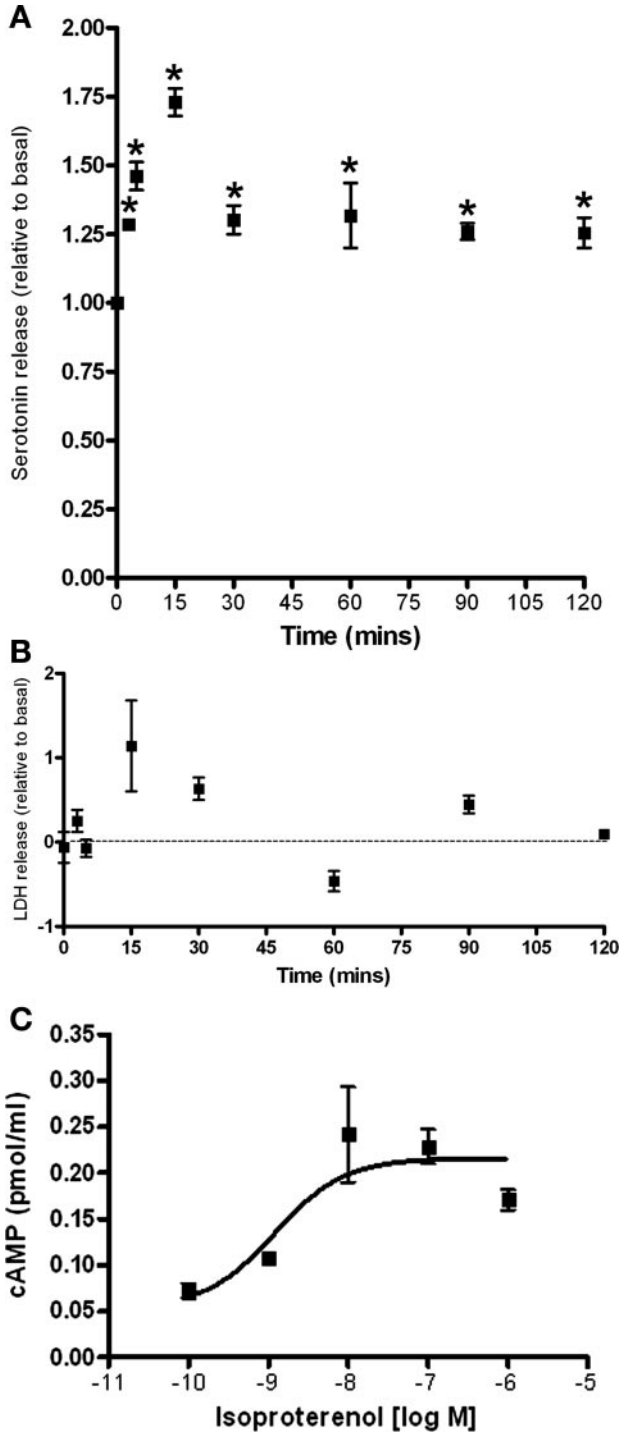

Fig. 6. Time course of isoproterenol (10^{-6} M)-stimulated serotonin release from 2-h cultured EC cells. *A*: significant stimulation was evident at 3 min, maximal at 15 min, and detectable for 2 h. *B*: no effect was noted on lactate dehydrogenase (LDH) release over this time period. *C*: intracellular cAMP measured at 15 min was dose dependently elevated by isoproterenol with an estimated EC_{50} of 1.2×10^{-9} M. Values are means ± SE for $n = 3$ (experiments performed in duplicate). *$P < 0.05$ vs. unstimulated cells.

inhibited by octreotide with an IC_{50} of 3×10^{-9} M (Fig. 7*D*). PACAP-38 stimulated serotonin secretion ($EC_{50} = 1.15 \times 10^{-9}$ M; Fig. 7*E*), but gastrin had no effect on EC cell serotonin secretion (Fig. 7*F*). Inhibitors of basal serotonin secretion included acetylcholine chloride ($IC_{50} = 3 \times 10^{-10}$ M; Fig. 7*G*) and $GABA_A$ ($IC_{50} = 2.9 \times 10^{-10}$ M; Fig. 7*H*). Isoproterenol-stimulated secretion (EC_{50}) was inhibited by acetylcholine chloride with an IC_{50} of 6×10^{-10}M (Fig. 7*I*) and by $GABA_A$ with an IC_{50} of 4×10^{-10} M (Fig. 7*J*). LDH levels were not altered by any of the evaluated ligands.

In separate studies, we assessed the effects of cell contamination (particularly D cells). Secretory responses to maximal concentrations of these agents were evaluated in cultured pre-FACS-sorted Nycodenz gradient-centrifuged cells (the F_N fraction). Serotonin secretion was stimulated in F_N cells by maximal concentrations of isoproterenol (1 μM, 40% above basal) and acetylcholine (1 μM, 30% above basal). In contrast, in AO-FACS-sorted cells, isoproterenol (1 μM)-stimulated serotonin secretion by 90% above basal, whereas both acetylcholic (1 μM) and $GABA_A$ (10 μM) inhibited serotonin secretion (35–55% below basal). The addition of octreotide (1 μM) significantly inhibited the effect of isoproterenol (~90% inhibition above basal).

The Naive EC Cell Transcriptome

We (26) previously determined the transcriptome of *Mastomys* gastric ECL cells using an Affymetrix approach (Mouse U74A chips). Using the same technique but with newer expression GeneChips (Mouse Expression Set 430 chips), we now determined the gene expression profile of AO (2 μM)-FACS-sorted *Mastomys* EC cells and demonstrated the feasibility of quantifying gene expression from FACS-sorted ileal NE cells. As a comparator, we measured gene expression in the IM. In our preliminary studies, we noted both a significantly lower yield of total RNA from ethanol-fixed TPH-FACS-sorted cells [<2 ng/μl (10^6 cells)] compared with AO-FACS-sorted cells [~200 ng/μl (10^6 cells)] as well as a higher 3′-to-5′ GAPDH ratio (>7 vs. <5 for AO-FACS-sorted cells) on test chips, making this preparation unsuitable for RNA studies. We therefore focused on measuring the transcriptome in AO-FACS-sorted cells using two techniques. Initially, we identified the genes categorized as present in the FACS preparations to define the transcriptome. As a secondary analysis, we also defined genes that were overexpressed in these preparations compared with the IM. Sample comparison was undertaken using DNA Chip Analyzer (dCHIP) version 1.3 to identify differentially altered genes using the lower 90% confidence bound of fold change (>1.2 fold), and data were analyzed using the unpaired *t*-test ($P < 0.05$). Genes that were present and overexpressed in the EC cell transcriptome are the principal focus of this study.

A total of 8,125 probe sets were present in the AO-FACS-sorted fractions vs. 6,759 in the IM scraping. The latter included marker genes expected for the spectrum of different intestinal cell types including villin (enterocytes), mucins (goblet cells), and defensins (paneth cells). Marker genes for NE cells (chromogranins) were also expressed. When the two databases were compared, 722 genes were identified as upregulated (levels >2-fold, $P < 0.05$) in the AO-FACS fraction and 1,210 genes were identified as significantly downregulated.

Highly expressed genes (>4-fold) in the AO-FACS-sorted fractions included CgA and CgB (>6-fold, $P < 0.005$), TPH (>5-fold, $P < 0.001$), $VMAT_1$ (>4.5-fold, $P < 0.01$), and the serotonin transporter (>6.4-fold, $P < 0.01$) (all markers of the

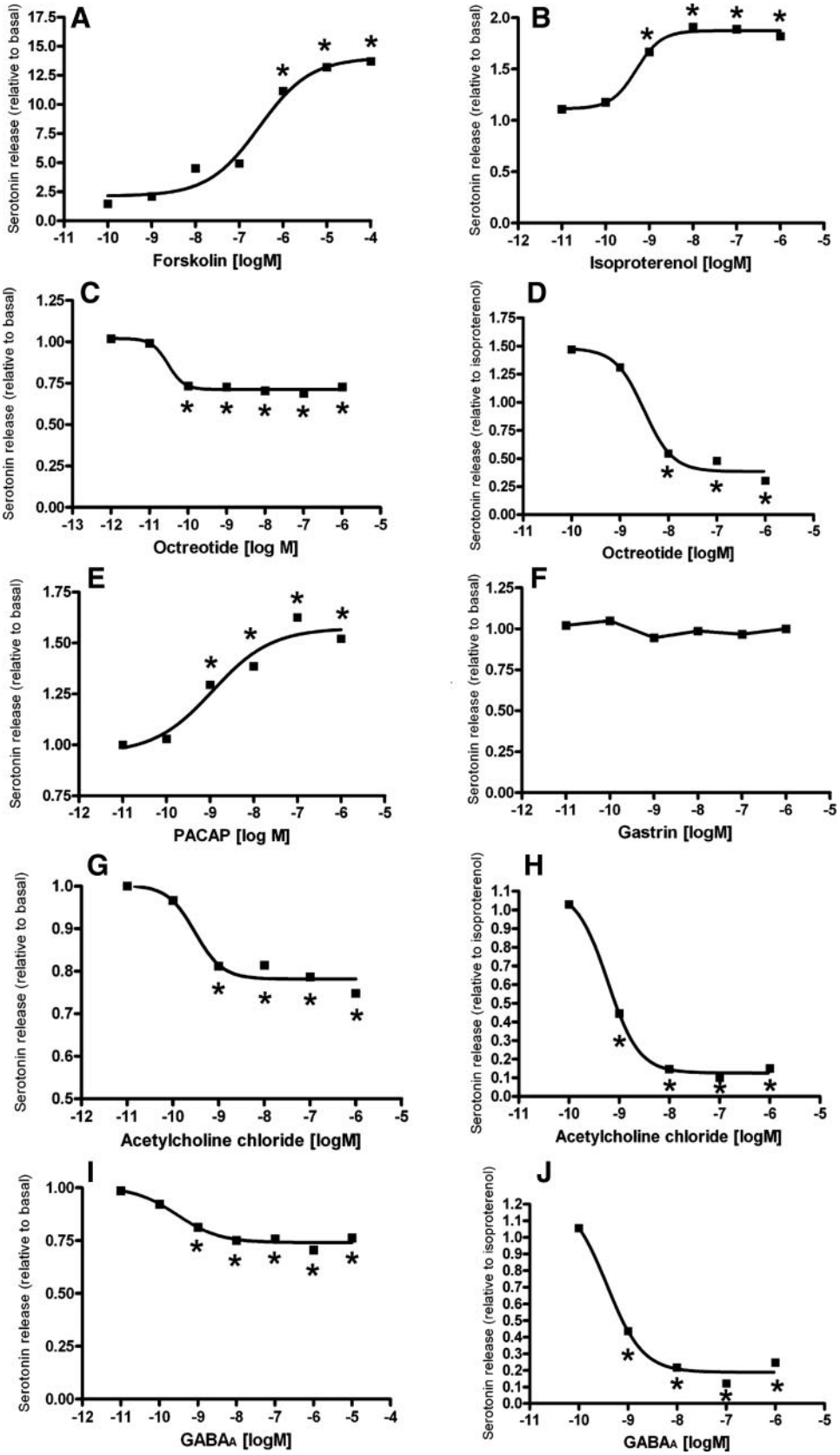

Fig. 7. *A–C*, *E–G*, and *I*: dose-response curves for serotonin secretion by forskolin (*A*), isoproterenol (*B*), octreotide (*C*), PACAP-38 (*E*), gastrin (*F*), ACh (*G*), and $GABA_A$ (*I*). *D*, *H*, and *J*: dose-response curves for inhibition of isoproterenol ($EC_{50} = 5 \times 10^{-10}$ M)-stimulated serotonin secretion by octreotide (*D*), ACh (*H*), and $GABA_A$ (*J*). With the use of these data, a half-maximal response (EC_{50} or IC_{50}) was calculated using nonlinear regression analysis. The EC_{50} values for forskolin, isoproterenol, and PACAP were calculated to be 5×10^{-10}, 4.5×10^{-10}, and 1.15×10^{-9} M. No effect was noted for gastrin. The IC_{50} values for octreotide, ACh, and $GABA_A$ were calculated to be 3×10^{-11}, 3×10^{-10}, and 2.9×10^{-10} M on basal secretion and 3×10^{-9}, 6×10^{-10}, and 3.7×10^{-10} M on isoproterenol-stimulated secretion. Data are presented as means for $n = 3$ (experiments performed in duplicate). *$P < 0.05$ vs. unstimulated cells (basal secretion).

Table 3. *Affymetrix GeneChip profiling of AO-FACS-sorted EC cells*

Gene	Expression/Type	Fold Elevation	*P* Value
	NE cell markers		
CgA and CgB	+	>64	<0.005
$VMAT_1$	+	>22.6	<0.01
TPH	+	>32	<0.001
Serotonin transporter	+	>6.4	<0.01
Guanylin	+	>4.6	<0.03
Substance P	+	>4.9	<0.02
	Receptors		
$GABA_A$	α_2 and β	>4	<0.01
Prostaglandin	E_1 and F_4	2.5–10	<0.014
Interleukin	7 and 12-β_1	4–16	<0.03
Androgen		2.5	<0.03
Platelet-activating factor	Receptor 2	6.5	<0.01
Tachykinin	Receptor 2	4.3	<0.02
	Growth factors and growth factor receptors		
FGF	FGF receptor 2	>4	0.01
IGF	IGF receptor I	>3.2	0.013
TGF-β	Activin receptor IIA	>4	0.0007
NGF	NGF-γ	>14.1	0.005
SCGF	SCGF	>16	0.02
	Olfactory receptors		
OR	72	>2.5	0.046
SLC	21 all and 39	8–51	<0.04
	Cell cycle markers		
PCNA	+	24.25	0.002
Cyclins	A_2, D_2, D_3, G, and H	3.7–42.2	<0.002
Cdks	2	3.2	0.03
Cyclin inhibitors	p16/p27	>3.5	0.02

Shown is a selection of 34 genes identified to be significantly overexpressed in the *Mastomys* EC cell fraction compared with the IM by MAS5.0. Fold elevation and *P* values were compared with those in the IM by MAS5.0 (Wilcoxon rank test). $VMAT_1$, vesicular monoamine transporter 1; TGF-β, transforming growth factor-β; NGF, nerve growth factor; SCGF, stem cell growth factor; OR, olfactory receptor; SLC, solute carrier.

EC cell; Table 3). Both substance P (>4.6-fold, $P < 0.03$) and guanylin (>4.9-fold, $P < 0.02$) were also expressed. Marker genes for other NE cells (SST, glucagon, and CCK) were absent. Neurotensin transcripts were variably expressed but present in two-thirds of the EC cell preparations. Receptors including SST_R2, SST_R5, and $VPAC_1$, but not PAC_1, CCK_2, or neurotensin receptors, were identified. Levels of marker genes for other cell types in the ileum including villin (epithelial); mucin-2, -3, -4, and -5 (goblet); the defensins (β_1, β_4, β_5, β_6, and β_8) (paneth); CCR9 and CD4 (T cells); and CD45 and genes encoding lymphocyte antigen complexes (lymphocytes) were all absent. These results indicate that the gene expression profile of the FACS-sorted preparations is consistent with that expected for a homogenous preparation of intestinal EC cells.

The receptor profile included the expression of cholinergic (M_4), adrenergic (β_1), dopaminergic (D_2), serotoninergic (5-HT_{2C}), GABA (A and B), and prostaglandin ($E_{1/4}$ and F) receptors with an absence of histaminergic receptor subtypes. Growth factor receptors included IGF-I, IGF-II, transforming growth factor (TGF)-β_1, and FGF2. Growth factors included nerve growth factor-γ, stem cell growth factor, and members of the PDGF, TGF-β, and EGF families. A few olfactory or amino acid transporter transcripts were noted, including OR72, SLC21a11, and SLC39. A number of cell cycle genes including A2, D2/3, G, H, I, and L as well as PCNA, cdk2, and the cyclin inhibitors $P16^{INK4A}$ and $P27^{KIP1}$ were identified. Markers of carcinoid malignancy including melanoma antigen family D2 (MAGE-D2) and metastasis-associated 1 (MTA1) were not identified in these cells (27), but the mitosis-associated transcript NAP1L1 was expressed. We also noted that heat shock proteins (heat shock proteins 10, 27, 70, and 84) were expressed in EC cell preparations. These genes are presumably associated with stress responses and potentially represent activation by the cell preparative technique (Nycodenz gradient centrifugation and FACS). This indicates that this subset of genes may therefore not be present in situ in the EC transcriptome.

DISCUSSION

In this work, we present the methodology to isolate homogenous preparations (>99%) of live ileal EC cells that can be maintained in short-term culture. We defined the presence of PACAP and SST receptors on ileal EC cells and identified the absence of gastrin CCK_2 receptors. In addition, in vitro secretory studies indicated that PACAP and SST, respectively, are capable of significant secretory stimulatory and inhibitory effects on serotonin secretion. Furthermore, our data support the proposal that the ileal EC cell is also subject to secretory modulation by neural agents including β-adrenergic, muscarinic, and GABA compounds. This information allows for the preliminary construct of a functional model of naive ileal EC cell serotonin secretion (Fig. 8).

In the past, methodology for the enrichment of impure rat ileal EC cells (~80%) has been reported (46). This methodology entailed isolation of rat ilea, enzymatic digestion, and EDTA chelation. The resulting crude mucosal sample was separated by counterflow elutriation and enriched by performing step density gradient centrifugation to yield an ~80% pure preparation. In our present study, we adopted a FACS technique to collect cells that specifically expressed the rate-limiting enzyme of serotonin synthesis (TPH), expressed a

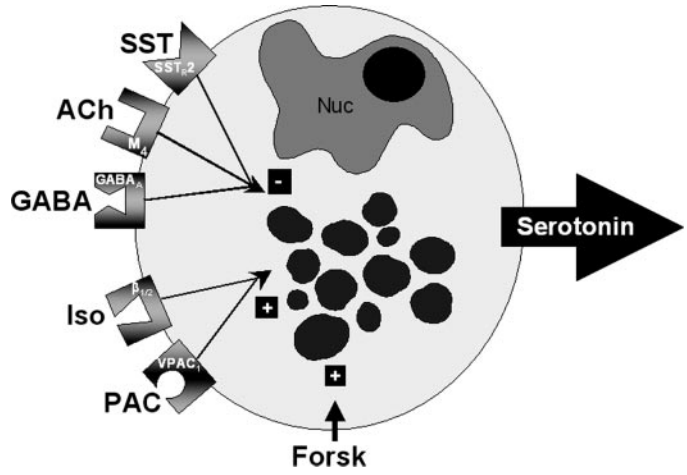

Fig. 8. Schematic representation of the potential regulation of serotonin release from EC cells. Inhibitors (−) of secretion include SST (SST_R2), ACh (M_4), and GABA ($GABA_A$). Stimulators (+) of secretion include isoproterenol (Iso; $\beta_{1/2}$-adrenergic) and PACAP ($VPAC_1$). Forskolin (Forsk) is a direct activator of serotonin release via activation of the adenylyl cyclase pathway. Nuc, nucleus.

PACAP receptor, or were AO positive and produced a 99% pure ileal EC cell preparation. All preparations were preenriched for ileal EC cells by Nycodenz gradient centrifugation (EC cell enrichment ~80%). The use of TPH immunostaining resulted in >98% positive preparations of ileal EC cells, fluoro-PACAP in >96% EC cells, and AO in >99% EC cells.

In addition to producing a homogenous preparation, we sorted live ileal EC cells by using either fluoro-PACAP or AO to prelabel these cells. Initially, PACAP-positive *Mastomys* ileal EC cells could not be identified using the same concentrations and conditions that were used to isolate gastric ECL cells (38). Increasing the fluoro-PACAP concentration 100-fold resulted in identification of a discrete sortable cell population. These studies were discontinued because *1*) cell preparations did not reach levels of homogeneity; *2*) the predominant high-affinity PACAP receptor (PAC_1) was not expressed on ileal EC cells; and *3*) prelabeling with this agent, which is known to induce gastric NE cell function (38), could potentially have the same effect on ileal EC cells, which would limit the validity of physiological studies.

Increasing concentrations of AO resulted in an easily detectable population of positive cells that could be sorted by FACS. However, this increase in fluorescence was negated by both a decrease in cell viability (from 94% to 57%, 10-fold increase in LDH measurements) and unstimulated serotonin release (a 5-fold decrease at 20 μM concentrations). On the basis of this information, a concentration of 2 μM was determined to be effective (nondeleterious) in producing functional ileal EC cells that could be cultured and examined in vitro. With the use of this strategy, serotonin secretory responses to a number of candidate neural and hormonal agents could be quantified and compared with the Nycodenz gradient-enriched fraction (~80% EC cells). This was undertaken to both quantitate serotonin secretion and establish the effect of contaminating NE cells on ileal EC cell function.

Serotonin secretion was specifically examined because prior studies have identified it as the dominant secretory product of the ileal EC cell and a key regulator of intestinal secretion and motility (10, 12, 20). Apart from the observation that serotonin is one of the main secretory products of ileal EC cells (8, 52), evidence has accumulated to suggest that it may be of clinical relevance in a number of gastrointestinal problems including diarrhea, motility abnormalities, and irritable bowel syndrome. (11). Of particular relevance is the observation that many intestinal carcinoid tumors are of EC origin and that serotonin is a key bioactive amine involved in the genesis of the carcinoid syndrome and thus implicated in the diarrhea and motility abnormalities that characterize this disease (34, 35). Plasma serotonin levels and urinary 5-hydroxyindoleacetic levels are regarded as the primary diagnostic criteria in the establishment of the diagnosis of carcinoid neoplasia (34). In addition, studies in humans have demonstrated that an infusion of 5-hydroxytryptophan (the upstream precursor of serotonin) induces diarrhea and abdominal cramping (22). Delineating the physiology of the ileal EC cell and its secretory regulators is thus of considerable scientific and clinical relevance.

In short-term culture, FACS-sorted ileal EC cells were stimulated to secrete serotonin by the adenylyl cyclase activator forskolin, demonstrating receptor-independent cAMP-mediated serotonin secretion (Table 4). The dual β_1-adrenoreceptor agonist and cAMP activator isoproterenol induced serotonin secretion within 3 min, suggesting that a rapid neurally mediated event might be a component of the ileal EC cell response profile. This would certainly be consistent with the sudden onset of "carcinoid crisis" and the associated massive release of serotonin engendered by a provocative epinephrine-mediated event (contrast injection, general anesthesia). Both serotonin secretion and cAMP release exhibited dose-dependent responses with EC_{50}s of 4.5×10^{-10} and 1.2×10^{-9} M, respectively. The time courses for secretion and cAMP activation are similar in this cell type (data not shown) and suggest that in ileal EC cells, β-adrenergic receptor activation (via at least the cAMP pathway) plays a role in serotonin secretion. This effect on secretion is similar to that on neurotensin release from canine ileal NT cells (2).

Table 4. *Stimulatory and inhibitory concentrations of select agents on EC cell serotonin release in vitro*

Agent	Stimulation (EC_{50}), M	Inhibition (IC_{50}), M	
		Basal release	Isoproterenol (5×10^{-10} M)-stimulated secretion
Forskolin	5×10^{-10}		
Isoproterenol	4.5×10^{-10}		
PACAP-38	1.2×10^{-9}		
Gastrin octreotide	None	3×10^{-11}	3×10^{-9}
Acetylcholine chloride		3×10^{-10}	6×10^{-10}
$GABA_A$		2.9×10^{-10}	3.7×10^{-10}

PACAP, pituitary adenylate cyclase-activating polypeptide.

The SST agonist octreotide dose dependently inhibited basal and isoproterenol (5×10^{-10} M)-stimulated serotonin secretion with IC_{50}s of 3×10^{-11} and 3×10^{-9} M, respectively (Table 4). The presence of the SST_R2 transcript was demonstrated in EC cells, suggesting that, physiologically, SST produced from adjacent SST-secreting cells may modulate serotonin secretion via binding to this receptor.

Two of the best-characterized regulators of gastric ECL cell histamine secretion, gastrin and PACAP (42, 45, 55), were next examined in the F_{FACS} fraction. Gastrin had no effect on ileal EC cell serotonin secretion, consistent with the demonstration of the absence of the CCK_2 transcript. A direct effect of gastrin on the ileal EC cell is thus unlikely. It is therefore possible that pentagastrin when used as a provocative test of carcinoid tumor amine secretion (34) produces its effect via an intermediate mechanism, which may include pentagastrin-stimulated release of adrenergic compounds (1). This is consistent with the observed serotonin responses initiated by the β-adrenergic mimetic isoproterenol, noted in the present study.

In contrast to gastrin, PACAP stimulated ileal EC cell serotonin secretion with an EC_{50} of 1.2×10^{-9} M. This effect is presumably mediated via the $VPAC_1$ receptor because transcripts for this PACAP receptor subtype but not PAC_1 were identifiable. The stimulatory effect of PACAP was in contrast to other studies using ex vivo preparations, which have demonstrated that PACAP (and VIP) inhibits serotonin release (16, 30). This reported difference in effects reflects the complexity inherent in the use of models that contain multiple cell systems and the likelihood of the activation of intermediate cellular regulatory circuitry. A similar experience was noted in dissecting out the effect of gastrin in isolated gastric ECL cells and isolated gastric parietal cells as opposed to the data that

emerged from the use of Ussing chamber gastric mucosa or isolated gastric glands (23). Overall, these may be an upstream effect whereby PACAP/VIP stimulates the release of an unidentified inhibitory agent (e.g., SST) in a fashion analogous to that reported in an isolated gastric cell study (23).

Acetylcholine chloride, an important regulator of cellular muscarinic responses, inhibited basal serotonin secretion from F_{FACS} cells with an IC_{50} of 3×10^{-10} M and inhibited isoproterenol (5×10^{-10} M)-stimulated serotonin secretion with an IC_{50} of 6×10^{-10} M (Table 4). Our observations of the inhibition of serotonin secretion are supported by the present GeneChip studies demonstrating muscarinic (M_4) receptors on these cells as well as the identification of M_2 and M_4 receptors in rat EC cell preparations (47). In the isolated canine NT cell studies of Barber et al. (2, 3), the muscarinic M_3 receptor agonist carbachol inhibited epinephrine-stimulated neurotensin secretion. This suggests that ileal NE secretion may share a common inhibitory pathway via muscarinic receptor activation.

GABA is a major inhibitory neurotransmitter in the mammalian nervous system (14) and functions through a paracrine or autocrine effect via $GABA_A$ receptor activation (13). More recently, it has become apparent that this agent may play a similar role as a signaling molecule in endocrine organs. Thus GABA and GABA receptors have been identified in peripheral endocrine organs, including growth hormone cells of the anterior pituitary lobe (17), in pancreatic islets (43), and in the testis of human and rodents (18). It is thought that GABA may regulate the synthesis and release of hormones in an autocrine/paracrine manner (13). In *Mastomys* ileal EC cells, $GABA_A$ dose dependently inhibited basal and adrenergic-stimulated serotonin secretion with IC_{50}s of 3×10^{-10} and 4×10^{-10} M, respectively. These observations are supported by our present GeneChip studies demonstrating the presence of ileal EC cell $GABA_A$ receptors as well as another report (47) of the identification of GABA receptors in rat EC cell preparations. These results are in contrast to an earlier study (46) that demonstrated no effect of $GABA_A$ on EC function in an 80% pure ileal EC cell preparation. The absence of an effect in the latter preparation may reflect a degree of cell heterogeneity in that it was a mixed preparation contaminated with a spectrum of cells from the IM (47). In the present study, a comparison of secretory responses in FACS-sorted ileal EC cells and cell preparations enriched by gradient centrifugation only (~80% pure) we noted that in the latter (impure) preparations both a lower serotonin secretory responses (~50% of FACS-sorted cells) as well as different responses (stimulation by acetylcholine and $GABA_A$ in contrast to inhibition) to physiological agents. These differences emphasize the limitations of an impure ileal EC cell preparation and confirm the physiological relevance of a homogenous cell preparation in the assessment of function.

The response of EC cells to $GABA_A$ raises the consideration of the efficacy of pharmacological targeting the $GABA_A$ receptor with specific classes of benzodiazepines that can stimulate $GABA_A$ receptor function to inhibit EC cell function.

Neither the SST transcript nor SST immunoreactivity could be demonstrated in the FACS-sorted preparations. In contrast, the F_N fraction expressed SST transcript and SST-containing cells were readily identifiable (~5% of the preparation). A comparison of secretory responses in F_{FACS} and F_N preparations demonstrated a number of marked differences. The stimulatory effect of a maximal dose of isoproterenol was greater than two times higher in the pure cell preparation compared with the F_N preparation, whereas no inhibitory effect of acetylcholine could be demonstrated in these cells. Inhibitory responses to maximal concentrations of $GABA_A$ and octreotide were noted in the F_N preparation, but these were not as marked as in the pure EC cell preparation. We interpreted this as a reflection of actively secreting SST cells (or an unknown other inhibitory cell type) that hinder serotonin release under short-term culture conditions in the F_N preparations. It would thus seem optimal to utilize pure preparations of EC cells to define the physiology of the EC cell.

Neurotensin, a peptide with a variety of putative biological functions of as-yet-unproven clinical significance, has also been noted to be involved in the regulation of intestinal motility (52). The identification of the neurotensin transcript in FACS-sorted preparations indicates that the N cell is a component of the <1% contaminating population. In the examination of the EC cell transcriptome, using the same approach that we used to define the transcriptome of *Mastomys* gastric ECL cells but with a more recent version of mouse chips (Mouse Expression Set 430 chips) (26), neurotensin receptors could not be identified. The absence of these receptors suggests that it is unlikely that the EC cell would respond to this peptide, and, hence, it seems doubtful that neurotensin might perturb EC functional assessment.

Further analysis of the FACS-sorted cell transcriptome revealed a receptor profile (SST, PACAP, acetylcholine, and GABA) consistent with the physiological results of our study. In addition, serotoninergic ($5\text{-}HT_{2C}$) and tachykininergic (TAC_2) but not histaminergic receptors were present on the EC cells, suggesting that these cells may be autoregulated by serotonin and substance P. The presence of olfactory receptors suggests that the EC cell may also have a defined sensing function particularly because EC cells have apical cytoplasmic processes that access the bowel lumen (49). Overall, the spectrum of growth factors and receptors identified is consistent with previous reports in small intestinal (EC-derived) carcinoids (37, 54), whereas the presence of cell cycle genes suggests that these cells may have the potential to proliferate and are not terminally differentiated as other specialized cells in the small intestine [e.g., epithelial cells (33)].

The definition of the EC cell transcriptome can also be utilized as a template to identify transcripts altered in malignant EC cells. Of note, therefore, is the fact that a number of marker genes that we have previously identified as being associated with malignant human EC cells (carcinoid tumors), particularly the adhesin MAGE-D2 and MTA1 (27), were absent in the naive *Mastomys* EC cell preparations. Elucidation of the naive human EC cell transcriptome will potentially *1*) identify novel markers of EC cell neoplasia and *2*) define pathways that are aberrantly expressed during neoplasia. We have previously used the delineation of the naive gastric ECL cell transcriptome as a template for the identification of neoplastic pathways in an animal model of gastric carcinoid disease (26).

In summary, we produced a homogenous preparation (>99%) of viable ileal EC cells from *Mastomys* that do not contain contaminating SST cells. The cells can be maintained

in short-term culture and exhibit receptors for PACAP and SST but not gastrin or histamine. They respond to adrenergic agents and PACAP with serotonin secretion via a mechanism that is at least in part cAMP mediated. Serotonin secretion was inhibited by octreotide, acetylcholine, and $GABA_A$. Gastrin has no effect on EC cell secretion. These studies, in 99% pure *Mastomys* EC cells, are consistent with studies of short-term cultured rat ileal enteroglucagon and neurotensin cells that secrete enteroglucagon-like and neurotensin-like immunoreactivity in response to the β-adrenergic agonist isoproterenol and the cAMP activator forskolin. In our studies, serotonin secretion was reversed by the muscarinic agent acetylcholine chloride and SST, a phenomenon shared with EG/L and NT cells (2, 6). These data suggest that NE cells of the ileum may represent a group of sensory and regulatory cells that are subject to common adrenergic, muscarinic, and SST modulating influences (Fig. 8).

In conclusion, these studies provide the methodology by which the rodent ileal EC cell can be defined and its responses to stimuli quantified. It is likely that the application of this methodology will enable the evaluation of the human ileal EC cell and an assessment of its biological function in both health and disease. Overall, these studies will allow for a precise characterization of the ileal EC cell and provide an opportunity to identify the regulatory pathways of this cell system and its role in small bowel physiology. In addition, it provides an opportunity to assess the potential role of the EC cell in complex and ill-understood diseases such as irritable bowel syndrome and refine understanding of EC cell-derived carcinoid tumors that have hitherto eluded precise cellular and biological assessment.

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REFERENCES

1. **Ahlman H, Nilsson O, Wangberg B, and Dahlstrom A.** Neuroendocrine insights from the laboratory to the clinic. *Am J Surg* 172: 61–67, 1996.
2. **Barber D, Buchan A, Walsh J, and Soll A.** Regulation of neurotensin release from canine enteric primary cell cultures. *Am J Physiol Gastrointest Liver Physiol* 250: G385–G390, 1986.
3. **Barber DL, Buchan AM, Walsh J, and Soll AH.** Isolated canine ileal mucosal cells in short-term culture: a model for study of neurotensin release. *Am J Physiol Gastrointest Liver Physiol* 250: G374–G384, 1986.
4. **Borin J, Tang L, Kidd M, Miu K, Bortecen K, Sandor A, and Modlin I.** Somatostatin receptor (SSTR) regulation of gastric enterochromaffin-like (ECL) cell transformation to gastric carcinoid. *Surgery* 120: 1026–1032, 1996.
5. **Brun P, Mastrotto C, Beggiao E, Stefani A, Barzon L, Sturniolo GC, Palu G, and Castagliuolo I.** Neuropeptide neurotensin stimulates intestinal wound healing following chronic intestinal inflammation. *Am J Physiol Gastrointest Liver Physiol* 288: G621–G629, 2005.
6. **Buchan AM, Barber DL, Gregor M, and Soll AH.** Morphologic and physiologic studies of canine ileal enteroglucagon-containing cells in short-term culture. *Gastroenterology* 93: 791–800, 1987.
7. **Buffa R, Capella C, Fontana P, Usellini L, and Solcia E.** Types of endocrine cells in the human colon and rectum. *Cell Tissue Res* 192: 227–240, 1978.
8. **Cetin Y, Kuhn M, Kulaksiz H, Adermann K, Bargsten G, Grube D, and Forssmann WG.** Enterochromaffin cells of the digestive system: cellular source of guanylin, a guanylate cyclase-activating peptide. *Proc Natl Acad Sci USA* 91: 2935–2939, 1994.
9. **Challacombe D and Wheeler E.** Possible neural projections from enterochromaffin cells. *Lancet* 2: 1502, 1983.
10. **Coates MD, Mahoney CR, Linden DR, Sampson JE, Chen J, Blaszyk H, Crowell MD, Sharkey KA, Gershon MD, Mawe GM, and Moses PL.** Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome. *Gastroenterology* 126: 1657–1664, 2004.
11. **Crowell MD.** Role of serotonin in the pathophysiology of the irritable bowel syndrome. *Br J Pharmacol* 141: 1285–1293, 2004.
12. **Crowell MD, Shetzline MA, Moses PL, Mawe GM, and Talley NJ.** Enterochromaffin cells and 5-HT signaling in the pathophysiology of disorders of gastrointestinal function. *Curr Opin Investig Drugs* 5: 55–60, 2004.
13. **Doepner R, Geigerseder C, Frungieri M, Gonzalez-Calvar S, Calandra R, Raemsch R, Fohr K, Kunz L, and Mayerhofer A.** Insights into GABA receptor signaling in TM3 Leydig cells. *Neuroendocrinology* 81: 381–390, 2005.
14. **Farrant M and Nusser Z.** Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. *Nature Rev Neurosci* 6: 215–229, 2005.
15. **Forgac M.** Structure and function of vaculoar class of ATP-driven proton pumps. *Physiol Rev* 69: 765–796, 1989.
16. **Fujimiya M, Yamamoto H, and Kuwahara A.** Effect of VIP and PACAP on basal release of serotonin from vascularly and luminally perfused rat duodenum. *Am J Physiol Gastrointest Liver Physiol* 275: G731–G739, 1998.
17. **Gamel-Didelon K, Kunz L, Fohr KJ, Gratzl M, and Mayerhofer A.** Molecular and physiological evidence for functional γ-aminobutyric acid (GABA)-C receptors in growth hormone-secreting cells. *J Biol Chem* 278: 20192–20195, 2003.
18. **Geigerseder C, Doepner R, Thalhammer A, Frungieri MB, Gamel-Didelon K, Calandra RS, Kohn FM, and Mayerhofer A.** Evidence for a GABAergic system in rodent and human testis: local GABA production and GABA receptors. *Neuroendocrinology* 77: 314–323, 2003.
19. **Gershon M.** Reveiw article: roles played by 5-hydroxytryptamine in the physiology of the bowel. *Aliment Pharmacol Ther* 13, *Suppl* 2: 15–30, 1999.
20. **Gershon M.** Review article: serotonin receptors and transporters—roles in normal and abnormal gastrointestinal motility. *Aliment Pharmacol Ther* 20, *Suppl* 7: 3–14, 2004.
21. **Harmar T and Lutz E.** Multiple receptors for PACAP and VIP. *Trends Pharmacol Sci* 15: 97–99, 1994.
22. **Haverback B and Davidson J.** Serotonin and the gastrointestinal tract. *Gastroenterology* 35: 570, 1958.
23. **Hersey S and Sachs G.** Gastric acid secretion. *Physiol Rev* 75: 155–189, 1995.
24. **Jordison M, Beales I, and Calam J.** Soybean agglutin stimulated cholecystokinin release from cultured abbit jejunal cells requires calcium influx via L-type calcium channels. *Peptides* 19: 1541–1547, 1998.
25. **Kellum J, Alberquerque F, Stoner M, and Harris R.** Stroking human jejunal mucosa induces 5-HT release and Cl^- secretion via afferent neurons and 5-HT4 receptors. *Am J Physiol Gastrointest Liver Physiol* 277: G515–G520, 1999.
26. **Kidd M, Hinoue T, Eick G, Lye KD, Mane SM, Wen Y, and Modlin IM.** Global expression analysis of ECL cells in *Mastomys natalensis* gastric mucosa identifies alterations in the AP-1 pathway induced by gastrin-mediated transformation. *Physiol Genomics* 20: 131–142, 2004.
27. **Kidd M, Modlin I, Mane S, Camp R, Eick G, and Latich I.** The role of genetic markers, NAP1L1, MAGE-D2 and MTA1, in defining small intestinal carcinoid neoplasia. *Ann Surg Oncol.* 13: 253–262, 2006.
28. **Kolby L, Wangberg B, Ahlman H, Modlin I, Granerus G, Theodorsson E, and Nilsson O.** Histidine decarboxylase expression and histamine metabolism in gastric oxyntic mucosa during hypergastrinemia and carcinoid formation. *Endocrinology* 137: 4435–4442, 1996.
29. **Lambrecht NW, Yakubov I, Scott D, and Sachs G.** Identification of the K efflux channel coupled to the gastric H-K-ATPase during acid secretion. *Physiol Genomics* 21: 81–91, 2005.

30. **Lauffer J, Modlin I, and Tang L.** Biological relevance of pituitary adenylate cyclase-activating polypeptide (PACAP) in the gastrointestinal tract. *Regul Pept* 84: 1–12, 1999.
31. **Lauffer J, Tang L, Zhang T, Hinoue T, Rahbar S, Odo M, Modlin I, and Kidd M.** PACAP mediates the neural proliferative pathway of *Mastomys* enterochromaffin-like cell transformation. *Regul Pept* 102: 157–164, 2001.
32. **Lauffer JM, Modlin IM, Hinoue T, Kidd M, Zhang T, Schmid SW, and Tang LH.** Pituitary adenylate cyclase-activating polypeptide modulates gastric enterochromaffin-like cell proliferation in rats. *Gastroenterology* 116: 623–635, 1999.
33. **Mariadson J, Nicholas C, L'Italien K, Zhuang M, Smartt H, Heerdt B, Yang W, Corner G, Wilson A, Klampfer L, Arango D, and Augenlicht L.** Gene expression profiling of intestinal epithelial cell maturation along the crypt-villus axis. *Gastroenterology* 128: 1081–1088, 2005.
34. **Modlin IM, Kidd M, Latich I, Zikusoka MN, and Shapiro MD.** Current status of gastrointestinal carcinoids. *Gastroenterology* 128: 1717–1751, 2005.
35. **Modlin IM, Lye KD, and Kidd M.** A 5-decade analysis of 13,715 carcinoid tumors. *Cancer* 97: 934–959, 2003.
36. **Moertel CG.** Karnofsky memorial lecture. An odyssey in the land of small tumors. *J Clin Oncol* 5: 1502–1522, 1987.
37. **Oberg K.** Expression of growth factors and their receptors in neuroendocrine gut and pancreatic tumors, and prognostic factors for survival. *Ann NY Acad Sci* 733: 46–55, 1995.
38. **Oh DS, Lieu SN, Yamaguchi DJ, Tachiki K, Lambrecht N, Ohning GV, Sachs G, Germano PM, and Pisegna JR.** PACAP regulation of secretion and proliferation of pure populations of gastric ECL cells. *J Mol Neurosci* 26: 85–98, 2005.
39. **Polak J, Pearse A, and Heath C.** Complete identification of endocrine cells in the gastrointestinal tract: use of the semithin-thin section technique to identify motilin cells in human and animal tissue. *Gut* 16: 225–229, 1976.
40. **Popesko P.** *A Colour Atlas of the Anatomy of Small Laboratory Animals*. London: Wolfe, 1992.
41. **Portela-Gomes G, Grimelius L, and Bergstrom R.** Enterochromaffin (argentaffin) cells of the rat gastrointestinal tract: an ultrastructural study. *Acta Pathol Microbiol Immunol Scand* 92: 83–89, 1984.
42. **Prinz C, Kajimura M, Scott D, Mercier F, Helander H, and Sachs G.** Histamine secretion from rat enterochromaffin-like cells. *Gastroenterology* 105: 449–461, 1993.
43. **Rorsman P, Berggren PO, Bokvist K, Ericson H, Mohler H, Ostenson CG, and PAS.** Glucose-inhibition of glucagon secretion involves activation of GABA A-receptor chloride channels. *Nature* 341: 233–236, 1989.
44. **Roth K and Gordon J.** Spatial differentiation of the intestinal epithelium: analysis of enteroendocrine cells containing immunoreactive serotonin, secretin and substance P in normal and transgenic mice. *Proc Natl Acad Sci USA* 87: 6408–6412, 1990.
45. **Sandor A, Kidd M, Lawton G, Miu K, Tang L, and Modlin I.** Neurohormonal regulation of rat ECL cell histamine secretion. *Gastroenterology* 110: 1084–1092, 1996.
46. **Schafermeyer A, Gratzl M, Rad R, Dossumbekova A, Sachs G, and Prinz C.** Isolation and receptor profiling of ileal enterochromaffin cells. *Acta Physiol Scand* 182: 53–62, 2004.
47. **Schafermeyer A, Zanner R, Gratzl M, Sachs G, and Prinz C.** Characterization of enterochromaffin cells isolated from the rat ileum. In: *Cell Biology of the Chromaffin Cell*, edited by Borges R and Gandia L. Madrid, Spain: Insituto Teofilo Hernando, 2004, p. 175–185.
48. **Sjolund K, Sanden G, Hakanson R, and Sundler F.** Endocrine cells in human intestine: an immunocytochemical study. *Gastroenterology* 85: 1120–1130, 1983.
49. **Solcia E, Capella C, Vassallo G, and Buffa R.** Endocrine cells of the gastric mucosa. *Int Rev Cytol* 42: 223–286, 1975.
50. **Tang LH, Luque EA, Efstathiou JA, Bortecen KH, Kidd M, Tarasova NI, and Modlin IM.** Gastrin receptor expression and function during rapid transformation of the enterochromaffin-like cells in an African rodent. *Regul Pept* 72: 9–18, 1997.
51. **Tesmer JJ, Dessauer CW, Sunahara RK, Murray LD, Johnson RA, Gilman AG, and Sprang SR.** Molecular basis for P-site inhibition of adenylyl cyclase. *Biochemistry* 39: 14464–14471, 2000.
52. **Thompson A, Keelman M, Thiesen A, Clandinin M, Ropeleski M, and Wild G.** Small bowel review: normal physiology. Part 2. *Dig Dis Sci* 46: 2588–2607, 2001.
53. **Thomson AB, Keelan M, Thiesen A, Clandinin MT, Ropeleski M, and Wild GE.** Small bowel review: diseases of the small intestine. *Dig Dis Sci* 46: 2555–2566, 2001.
54. **Wulbrand U, Wied M, Zofel P, Goke B, Arnold R, and Fehmann HC.** Growth factor receptor expression in human gastroenteropancreatic neuroendocrine tumors. *Eur J Clin Invest* 28: 1038–1049, 1998.
55. **Zeng N, Athmann C, Kang T, Lyu RM, Walsh JH, Ohning GV, Sachs G, and Pisegna JR.** PACAP type I receptor activation regulates ECL cells and gastric acid secretion. *J Clin Invest* 104: 1383–1391, 1999.