Dopaminergic characteristics of isolated parietal cells from rats

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Summary — Recently we have identified a dopamine-producing system in the gastric mucosa of rats. All the available morphological data suggest that parietal cells synthesize dopamine. In the present study we investigated the dopaminergic characteristics of isolated parietal cells by different methods. Mixed gastric mucosal cells were isolated and size-fractionated by elutriation. The proportion of neurons, parietal and endocrine cells in the fractions were determined by immunocytochemistry (ICC) using antibodies to neurofilament, proton pump and chromogranin A, respectively. No neurons were found in any of the cell preparations, while 56% parietal cell and 0.0% endocrine cell were achieved in the parietally enriched fraction. By Western blot, a tyrosine hydroxylase (TH, the rate-limiting enzyme of the catecholamine synthesis) immunoreactive protein species was demonstrated in isolated mucosal cells, comigrating with the TH immunoreactivity from PC12 cells. The TH immunoreactivity was colocalized to parietal cells by ICC. Dopamine transporter (DAT), a regulator of extracellular/intracellular dopamine balance in the nervous system, was also demonstrated in parietal cells. A significant amount of dopamine and DOPA were measured by HPLC (13.4 and 9.57 pg/10⁶ cell, respectively) in parietally enriched cell fraction. Since this enriched cell fraction was virtually clear of both neurons and endocrine cells, demonstration of TH enzyme, DAT and dopamine in this fraction confirms that the parietal cell population might be a major source of dopamine in the rat stomach, supporting our previous results achieved using whole tissue samples.

dopamine / gastrointestinal system / parietal cell / cell isolation / immunocytochemistry

Introduction

The role of dopamine in gastrointestinal pathology has been studied since the mid-sixties (Häggendal, 1967; Häkanson and Owman, 1967; Häkanson et al, 1969; Cannata et al, 1979) with special regard to stress-related mucosal lesions (Glavin and Dugani, 1987; Glavin et al, 1991; Glavin and Hall 1994b). Dopamine was demonstrated in the gastric juice of man after stimulation of gastric acid secretion (Häggendal, 1967). Dopamine D1 receptor agonists reduced gastric acid secretion and increased bicarbonate and mucus secretion in the duodenum (Glavin, 1994; Flemström and Safsten, 1994). The effect of dopamine antagonists and agonists on experimental gastric ulcer generation and healing, respectively, have been reported in numerous papers (Szabó, 1979; Szabó et al, 1982; Glavin, 1989; MacNaughton and Wallace, 1989; Glavin and Szabó, 1990, 1991, 1993, Sikiric et al, 1991; Glavin and Hall 1994a, 1995; Glavin 1995a, b).

Two sources of gastric dopamine have been proposed: enterochromaffin-like cells (Larsson 1980; Häkanson et al, 1986) and sympathetic neurons (Schultzberg et al, 1980; Gräfner et al, 1985). However, the small number of enterochromaffin-like cells and the very sparse sympathetic innervation of the mucosa suggest another dopamine-producing compartment in the stomach.

Recently we reported that rat parietal cells show all the characteristics of known catecholaminergic cells (manuscript submitted). We found very high amounts of dopamine in the gastric juice after pyloric ligation. We also demonstrated a functionally active tyrosine hydroxylase enzyme (TH, the rate-limiting enzyme of catecholamine synthesis), the dopamine storage and the re-uptake capability of parietal cells (the presence of vesicular monoamine transporters and dopamine transporter, respectively) by immunohistochemistry, in situ hybridization histochemistry, Western and Southern blot techniques and ³H-dopamine uptake assays. We thus suggested that parietal cells may release dopamine in the rat stomach.

To further confirm the localization of dopaminergic markers to parietal cell, we have studied the dopaminergic characteristic of isolated, size-fractionated parietal cells to exclude any neuronal or endocrine contribution. We looked for the presence of TH enzyme in mixed or fractionated gastric mucosal cells by Western blot technique. We also investigated the presence of TH enzyme and dopamine transporter (DAT) in the fractionated parietal cells by immunocytochemistry. Finally, we measured the dopamine and DOPA content of isolated and fractionated gastric mucosal cells by HPLC.
Materials and methods

Mucosal cell isolation and fractionation

Buffers

Medium A (proteolytic mixture (mmol/L)): NaCl, 80; KCl, 5.0; CaCl$_2$, 1.0; MgCl$_2$, 1.5; Na$_2$HPO$_4$, 0.5; NaHPO$_4$, 1.0; NaHCO$_3$, 20.0; N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES)-Na, 50.0; D-(+)-glucose, 11.0 (all from JT Baker Chemical Co, Phililsburg, NJ), 2% bovine serum albumin (BSA) (fraction V, ICN Biomedicals, Aurora, OH), 2 mglmL protease type XXV (pronase E) (Sigma, St Louis, MO).

Medium B (dispersing and elutriation medium): medium A without CaCl$_2$, MgCl$_2$ or pronase E, only 0.1% BSA, containing 2.0 mMol ethylenediaminetetraacetic acid (EDTA)-Na$_2$ and 100 mg/mL deoxyribonuclease (DNase) (Sigma).

Medium C (washing medium): medium A without pronase E, only 0.1% BSA, containing 100 mg/mL DNase.

Cell dispersion

Gastric mucosal cells were isolated by a modification of Lewin’s reported sac method (Lewin et al, 1982; Nagy et al, 1994). Briefly, 3-5 adult, male Sprague-Dawley rats (Taconic Farms, Germantown, NY; 250-300 g) per experiment were killed by decapitation under anesthesia (80 mg/kg pentobarbital sodium ip; Anpro Pharmaceutical, Arcadia, CA). The stomachs were rapidly removed and opened at the cardia. The animals were anesthetized under anesthesia (80 mg/kg pentobarbital sodium ip; Anpro Pharmaceutical, Arcadia, CA). The stomachs were rapidly removed and opened at the cardia. The stomachs were everted by a glass rod. The stomach sacs were incubated in 50 mL proteolytic mixture (medium A). The rumen was discarded. Filled stomach sacs were washed by 0.9% NaCl, closed with a barbed silk ligature, and resuspended in 1 mL of medium C at 4°C. We refer to these unfractionated isolated mixed cells in 50 mL of medium B, collected by centrifugation (5 min at 400 g) and suspended in 1 mL of medium C.

Immunocytochemistry

Buffers

Phosphate buffered saline (PBS) (pH 7.4) (BioWhittaker, Walkersville, MD); 0.1 M Tris-HCl (pH 8.0) (Quality Biological, Gaithersburg, MD); Tris buffered saline (TBS): 0.1 M Tris-HCl, 0.15 M NaCl in distilled water (pH 8.0); BSA diluent: 1% bovine serum albumin, 0.6% v/v% Triton X-100 (Research Products International, Elk Grove Village, IL) in PBS (pH 7.4); paraformaldehyde fixative: 4% paraformaldehyde (Polysciences, Warrington, PA) in PBS (pH 7.4). Unless otherwise noted, all the immunocytochemical procedures were performed at room temperature (RT, ~20°C).

Cell mounting

Mixed or fractionated cells (5–20 x 10$^4$ in 50 mL medium B) were loaded into the chambers of a cytocentrifuge (Shandon, London, UK) and centrifuged onto silanized microslide slides (non-fixed cells). In other experiments, 2–10 x 10$^4$ cells were pelleted, and fixed with 4% formaldehyde in PBS fixative for 10 min at 4°C. After a wash in PBS, the cells were cryoprotected in 5% (2 h), 10% (4 h) and 20% (overnight, 4°C) sucrose (JT Baker Chemical Co, Phillipsburg, NJ) in PBS (pH 7.4) solutions, collected by centrifugation, mixed with 1 mL of Tissue-Tek OCT Compound (Miles Inc, Elkhart, IN) and frozen on dry ice. 10 µm thick sections were cut from mounted frozen cells in a cryostat (Frigocut-E 2800, Reichert-Jung, Heidelberg, Germany). The sections were thaw-mounted and air-dried at 37°C onto silanized slides, frozen, and stored at –80°C until used (fixed cells).

Immunocytochemical procedures

The conventional and amplified immunofluorescent and immunoperoxidase stainings have been previously described in detail (Hunyady et al, 1996). The primary antibodies used in the study are listed in table I. In conventional immunofluorescent stainings we used indocarboxyanine (Cy3, appears red through a fluorescent filter for rhodamine) or fluorescein isothiocyanate (FITC, green through a filter for FITC) conjugated secondary antibodies (Fab'2 fragments, Jackson ImmunoResearch, West Grove, PA) to either mouse or rabbit IgGs depending on the primary antibody. In fluorescent double stainings a secondary primary antibody from a different host species and a different fluorochrome-labeled secondary antibody were used to recognize the second antigen. In immunostainings 4',6-diamidino-2-phenylindole (DAPI) (Sigma) was used as a counterstain appearing in blue through an ultraviolet fluorescent filter. Fluorescent labeling was viewed with a fluorescent microscope (Leitz Dialux 20, Germany).

In fluorescent amplifications (Hunyady et al, 1996) the FITC labeled secondary antibody was followed by an anti-FITC horseradish peroxidase (HRP) conjugated tertiary antibody (Boehringer Mannheim, Indianapolis, IN; 1:1000 in BSA diluent). Tyramide-FITC or tyramide-rhodamine substrate for the HRP enzyme was the final amplified fluorescent reagent (TSA Renaissance KIT, DuPont NEN, Wilmington, DE).
Fig 1. Frequency of proton pump immunoreactive parietal cells (A, B, C) in mixed mucosal cells (A, D), endocrine-enriched (fraction F1, B, E) and parietal-enriched (fraction F4, C, F) isolated cells. Right panels D, E and F demonstrate the total cell numbers corresponding to the left panels A, B and C, respectively, using the nuclear marker, 4',6-diamidino-2-phenylindole. Parietal cells (arrows) were immunostained with a monoclonal antibody to proton pump. Note that the 20% parietal cell ratio in mixed cells (A, B) was reduced below 10% in fraction F1 (C, D) and increased over 55% in fraction F4 (E, F). Scale bars: 100 μm.
Fig 2. Frequency of chromogranin A immunoreactive endocrine cells (A, B, C) in mixed mucosal cells (A, D), endocrine-enriched (fraction F1, B, E) and parietal-enriched (fraction F4, C, F) isolated cells. Right panels D, E and F demonstrate the total cell numbers corresponding to the left panels A, B and C, respectively, using the nuclear marker, 4',6-diamidino-2-phenylindole. Endocrine cells (arrowheads) were immunostained with an antibody to chromogranin A. Note that the 2–3% endocrine cell ratio in mixed cells (A, B) was increased over 10% in fraction F1 (C, D) and reduced virtually to zero in fraction F4 (E, F). Scale bars: 100 μm.
Tyrosine hydroxylase (TH) immunoreactive protein in the isolated gastric mucosal cells. The Western blot demonstrates a TH immunoreactive protein species in mixed mucosal cells using the Boehringer-Mannheim antibody (table 1). The band corresponds in size to that in the adrenal gland used as a control. Western blots with three other antibodies to TH (table 1) showed the same molecular mass species in mixed mucosal cells. Apparent molecular masses indicated at left were estimated using pre-stained molecular mass markers.

In the immunoperoxidase stainings the primary antibody was followed by a biotin conjugated secondary antibody (Vector Labs., Burlingame, CA; I:1000 in BSA diluent). After the biotin conjugated secondary antibody, we used an avidin-biotin-peroxidase complex (ABC reagent, Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA) at dilutions of I:250 'A' and I:250 'B' in BSA diluent. The peroxidase activity was demonstrated using the 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate (40 mg% DAB, 0.0016% H2O2 in 0.1 M Tris-HCl (pH 8.0), prepared freshly and used for 7–10 min). Giemsa stain was used for counterstaining (Sigma).

Negative controls included incubations in non-immune mouse IgG or normal rabbit serum instead of the primary antibody, and leaving out the primary and/or the secondary antibodies.

After the immunostainings the sections were washed in distilled water, air dried, and coverslipped using Cytoseal 60 mounting medium (Stephens Scientific, Riverdale, NJ).

**Immunocytotoxic quantitation of cell fractionation**

Immunocytotoxic methods were used to evaluate the proportion of parietal cells and endocrine cells (using the antibody against proton pump and chromogranin A, respectively) in the cell fractions analyzed in Western blots and catecholamine measurements. The percentage of immunopositive cells from 300–1000 total cells was calculated from each fraction from protocols A and B.

**Western blots**

Either 20 μg protein from mixed mucosal cells or 2 μg protein from adrenal gland (positive control) was size-fractionated and electrophoretically loaded onto a Hybond-Enhanced Chemiluminescence nitrocellulose membrane (Amersham, Arlington Heights, IL). In another experiment, 106 fractionated mucosal cells (F0–F6) was loaded, and PC12 cells (positive) and human embryonic kidney cells (HEK293, negative) were used as controls. The buffers, the conditions and the immunoblot analysis using four different antibodies against TH (table 1) were described in our previous study (Mezey et al. 1996).

**Measurement of catecholamines**

Dopamine and DOPA concentrations of mixed and fractionated cells were measured by high pressure liquid chromatography with electrochemical detection after alumina extraction (Eisenhofer et al. 1986). Intra-assay coefficient of variations were 8.1% for dopamine, 3.9% for DOPA. For the measurements 1–10 x 10⁶ mixed or fractionated (F0–F4) cells were collected by centrifugation and extracted with 150 μL 1N perchloric acid. Protein precipitates were removed by centrifugation for 5 min at 15,000 g and the resulting supernatants were measured for catecholamines.

### Table 1. Characteristics of primary antibodies used in the study.

<table>
<thead>
<tr>
<th>Antigen (antibody abbreviation)</th>
<th>Host</th>
<th>Dilution</th>
<th>Source (reference)</th>
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<tr>
<td>Proton pump (HK)</td>
<td>Mouse</td>
<td>1:1000</td>
<td>Smolka and Swiger, 1992</td>
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<tr>
<td>Chromogranin A (ChrGr)</td>
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<td>Reinecke et al., 1991</td>
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<tr>
<td>Neurofilament M (NF)</td>
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<td>1:2000</td>
<td>Harris et al., 1991</td>
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<tr>
<td>Dopamine transporter (DAT)</td>
<td>Rabbit</td>
<td>1:2000</td>
<td>Vaughan, 1995</td>
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<td>Ziller et al., 1994</td>
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<tr>
<td>Tyrosine hydroxylase (Thi-TH)</td>
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<td>Chiodo and Kapatos, 1992</td>
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<td>Tyrosine hydroxylase (BM-TH)</td>
<td>Mouse</td>
<td>1:500</td>
<td>Rohrer et al., 1986</td>
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Results

Cell fractionation

The efficiency of cell fractionation is expressed as the proportion of parietal cells (fig 1) and endocrine cells (fig 2) in the different cell fractions as determined by immunocytochemistry. An order of magnitude difference in the immunopositive cell percentage between the endocrine-enriched versus the parietally enriched fractions (f0 vs f5 from protocol A or F1 vs F4 from protocol B) was achieved in both cases (parietal cell: 8 vs 75% or 5 vs 57%; endocrine cell: 8.6 vs 0.0% or 9.8 vs 0.0%, respectively). The actual proportions of the two cell types in the particular cell fractions are displayed in the figures for Western blots (fig 4) and catecholamine measurements (fig 7). No neurons were found in the preparations of either mixed or fractionated cells as determined using an antibody against neurofilament M (not shown).

We have identified an immunoreactive protein species of the same apparent molecular mass in the homogenate of adrenal gland and in mixed mucosal cells using four different antibodies to TH (fig 3, table I). We were also able to demonstrate a TH immunoreactive protein species in all fractions of mucosal cells from protocol A, even in fraction f5 which was virtually devoid of endocrine cells (fig 4). A high level of TH immunoreactive protein was found in PC12 cells, while TH immunoreactive protein was not detected in HEK293 cells. The shift of the TH immunoreactive protein species to a lower apparent molecular mass (compared to PC12 cells) in these elutriated fractions might be the consequence of proteolytic degradation during the 3-h elutriation procedure. No TH immunoreactive protein species with this lower apparent molecular mass were found in mixed mucosal cells, which were not subjected to elutriation (fig 3).

Table 1

<table>
<thead>
<tr>
<th></th>
<th>f6</th>
<th>f5</th>
<th>f4</th>
<th>f3</th>
<th>f2</th>
<th>f1</th>
<th>f0</th>
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<tr>
<td>Parietal cell (%)</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endocrine cell (%) (100)</td>
<td>-</td>
<td>0.4</td>
<td>0.0</td>
<td>0.1</td>
<td>0.6</td>
<td>1.2</td>
<td>4.1</td>
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</table>

Fig 4. Tyrosine hydroxylase (TH) immunoreactive protein in the fractionated gastric mucosal cells. The Western blot demonstrates the same TH immunoreactive protein species in each fraction from protocol A using the antibody from Boehringer-Mannheim (table I), even in the fraction virtually devoid of endocrine cells (f5). No TH immunoreactive protein was found in HEK293 cells used as a negative control. The intensity of the band in the lane of PC12 cells may refer to the high amount of TH protein in these cells. The shift of the TH immunoreactive protein species to a lower apparent molecular mass (compared to PC12 cells) in the elutriated mucosal cells might be the consequence of proteolytic degradation during the 3-h elutriation procedure. Western blots with the other three antibodies to TH in table I showed the same molecular mass species in the cell fractions. Apparent molecular mass indicated at left was estimated using pre-stained molecular mass markers.
Immunocytochemistry

Using four different antibodies against TH and the immunohistochemical signal amplification method (Hunyady et al., 1996) virtually all the proton pump immunoreactive parietal cells were also TH positive (fig 5). The rare TH positive non-parietal cells seen in the mixed cell preparations and in the endocrine cell rich fractions (F1 and F2) might represent catecholaminergic endocrine cells in the stomach.

All the parietal cells were immunoreactive for DAT, in addition to many non-parietal DAT positive cells (fig 6). These latter cells looked different in size and shape, suggesting different DAT-positive cell populations in the gastric mucosa.

Dopamine measurements

In the mixed or fractionated cell preparations from the representative example of protocol B we detected a significant amount of dopamine and DOPA (fig 7). In both endocrine and parietal cell rich fractions (F1 or F4) we measured a relatively high amount of dopamine (5.6 and 13.8 pg/10⁶ cell, respectively) and DOPA (2.2 or 9.7 pg/10⁶ cell, respectively). The parietal cell-rich fraction of F4 was virtually devoid of endocrine cells and neurons as potential sources of catecholamines, and still contained a comparable amount of dopamine and DOPA.

Discussion

The presented data support our conclusions established using tissue preparations: parietal cells of the rat stomach produce dopamine. The Western blot and the dopamine data on fractionated cells convincingly demonstrate that in the absence of any neuronal source and independently from some potentially dopaminergic endocrine cells there is both TH protein and dopamine in gastric mucosal cells. Since the majority of the cells in fractions F5 and F4 were parietal cells (75% and 57%, respectively), and since only the parietal cells showed TH immunoreactivity in these fractions, they represent a non-neuronal, non-endocrine source of dopamine in the rat stomach.

Simultaneous secretion of dopamine with the potentially harmful hydrochloric acid from parietal cells may be a self-protective mechanism in the stomach. Dopamine would most likely be very stable in the acidic milieu and therefore, probably has a relatively long half-life there. Secreted into the gastric pits, it could easily reach all the different cells in the gastric glands, and may act as a paracrine
hormone on dopamine D1b receptors, the mRNA of which has been demonstrated in most epithelial cells of the stomach (manuscript submitted). In the parietal cells, themselves, dopamine may act by an autocrine mechanism to reduce acid secretion. In other cell types dopamine may mediate different protective effects in the gastrointestinal system, such as stimulation of mucus and bicarbonate secretion (Glavin 1989; Flemström and Safsten, 1994). Dopamine may also communicate with other regulatory elements of the stomach, including immune cells, endocrine cells and the enteric nervous system.

The presence of dopamine transporter (DAT) in different cell types of the gastric mucosa may help to maintain a regulated extracellular dopamine concentration in the stomach. In addition to parietal cells, other types of cells may be able to remove dopamine from the extracellular compartment, including the gastric lumen. Based on the immunohistochemical data, one candidate for the removal of dopamine from the extracellular compartment is the vascular endothelium, most likely veins, where most of the dopamine may get metabolized. Carried by the portal vein, the remaining dopamine might be metabolized and inactivated in the liver, thus preventing the effects of high dopamine concentrations in the systemic circulation. Since many DAT-positive cells were not parietal cells, other cell types such as mucous cells, chief cells and endocrine cells might also contain DAT. All these cells might be able to re-uptake and then release dopamine by reversing the transport of dopamine in certain conditions as has been suggested to occur in neurons (Sulzer et al. 1993; Eshleman et al., 1994).

Protective effects of dopamine and dopaminergic compounds in the stomach are reported in different papers (Glavin, 1994; Flemström and Safsten, 1994), however, the source of dopamine has never been critically evaluated. Our findings demonstrate that parietal cells may represent a relevant source of dopamine in the rat stomach, possibly in addition to a local endocrine and neuronal source. Pathological depletion of the endogenous protective com-
Fig 7. Dopamine and DOPA content of mixed and fractionated gastric mucosal cells. The catecholamine content measured by HPLC is scaled as pg/10⁶ cells on the left Y axis. Percentages of parietal cells (scaled on right Y axis) and endocrine cells (scaled on left Y axis) in cell fractions from protocol B were assessed by counting the immunoreactive cells from 300–1000 total cells. Significant amounts of dopamine and DOPA were measured in the fractions enriched for endocrine cells (F1) and parietal cells (F4). In the fraction containing predominantly parietal cells (F4) there were very few or no endocrine cells and no neurons, suggesting association of dopamine with parietal cells.

pound, dopamine, may be a factor in different diseases of the GI tract, ie ulcerogenic property of Helicobacter pylori. On the other hand, stimulation of endogenous mucosal dopamine production and/or dopaminergic agonists might be beneficial in ulcer treatment in the future.

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References


Glavin GB (1993) Dopamine D1 receptor activation reduces experimentally-induced gastritis in rats. Gen Pharmacol 24, 1481–1482


Glavin GB (1995a) The antisecretory effects of clozapine, a dopamine D3 receptor antagonist, are blocked by the dopamine D1 receptor antagonist, SCH23390. Life Sci 56, PL365–368


