A novel nonneuronal catecholaminergic system: Exocrine pancreas synthesizes and releases dopamine

(monoamine transporters/mucosal healing/dopamine receptor)

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ABSTRACT Cells of the exocrine pancreas produce digestive enzymes potentially harmful to the intestinal mucosa. Dopamine has been reported to protect against mucosal injury. In looking for the source of dopamine in the small intestine, we found that the duodenal juice contains high levels of dopamine and that the pancreas itself has a high dopamine content that does not change significantly after chemical sympathectomy. Furthermore, we were able to demonstrate tyrosine hydroxylase (TH) activity in control pancreas as well as in pancreas from rats after chemical sympathectomy. Immunostaining and in situ hybridization histochemistry confirmed both the presence of TH, dopamine, and the dopamine transporter, and the mRNAs encoding TH and dopamine transporter, and the presence of both types of vesicular monoamine transporters in the exocrine cells of the pancreas. Since there are no catecholaminergic enteric ganglia in the pancreas, the above results indicate that pancreatic cells have all the characteristics of dopamine-producing cells. We suggest that the pancreas is an important source of nonneuronal dopamine in the body, and that this dopamine has a role in protecting the intestinal mucosa and suggests that dopamine D1b receptor agonists might be used to help mucosal healing in the gastrointestinal tract.

Dopamine protects against both gastric and intestinal mucosal injury. For instance, gastric and duodenal ulcers heal significantly faster after administration of dopamine agonists (1-4). However, the possible source of dopamine in the gastrointestinal system remained to be determined.

The exocrine pancreas produces and secretes digestive enzymes and bicarbonate and releases them into the duodenum, while endocrine cells in the islets of Langerhans synthesize and release hormones (such as insulin, glucagon, etc.) and are embedded in the exocrine pancreas. If dopamine in fact plays a protective role in the duodenum, then corelease with digestive enzymes from the exocrine pancreas seems reasonable. In the cells that are outside of the central nervous system, dopamine is generally considered to be a precursor of norepinephrine and epinephrine. Recent studies in swine (5), however, suggest that the mesenteric organs produce about half of total body dopamine and that dopamine in the mesenteric organs must not be exclusively a precursor to norepinephrine. The source of this dopamine is still unknown. We have recently discovered that the acid-secreting parietal cells of the stomach synthesize and release dopamine into the gastric lumen, where it may act as a paracrine hormone at dopamine receptors on epithelial cells (unpublished results). To determine if a similar mechanism exists in other parts of the digestive system, we analyzed both the pancreatic/duodenal secretions for the presence of dopamine and the pancreas itself for dopaminergic markers.

METHODS

Sample Collection. To collect pancreatic/duodenal juice from rats, we ligated the duodenum near the pylorus to eliminate the contribution of dopamine from the stomach and ligated the duodenum below the papilla Vateri (the opening of the pancreatic duct into the duodenum) and the ductus choledochus (where bile enters the pancreatic juice) in anesthetized rats. After 4 hr, the juice from the pancreas was taken and frozen until further processing. Collected from anesthetized rats, pancreatic tissue was frozen on dry ice and kept at −80°C until further processing.

Catecholamine Measurements. Concentrations of dopamine, dihydroxyphenylalanine (dopa), and 3,4-dihydroxyphenylacetic acid (DOPAC) were determined by liquid chromatography with electrochemical detection after alumina extraction (6). Intraxass coefficients of variation were 8.1% for dopamine and 3.9% for DOPAC.

Chemical Sympathectomies (CSs). CSs of adult male Sprague–Dawley rats were performed using 6-hydroxydopamine (6-OHDA), according to the following schedule. To avoid the initial side effects due to quick depletion of peripheral norepinephrine terminals, rats were first given 6-OHDA at a low dose (5 mg/kg) intraperitoneally. After 12 hr, each rat received 15 mg/kg, and then two additional doses of 30 mg/kg at 24 and 36 hr. Control rats received injections of vehicle on the same schedule. Four days after the last injection, the rats were anesthetized, the pylorus was ligated, and samples were collected as above. Norepinephrine content of the heart was used to control the completeness of the CS. All treated rats showed >95% reduction of cardiac noradrenaline levels.

Immunohistochemistry. For immunohistochemistry, rats were anesthetized with pentobarbital sodium (40 mg/kg body weight), then perfused with 4% paraformaldehyde. The pancreas were removed, cryoprotected in 20% sucrose, frozen on dry ice, and cut in a cryostat onto silanized slides in 12-μm-thick sections. To decrease nonspecific staining, the sections were pretreated for 30 min at room temperature (RT) in a solution containing 0.6% Triton X-100, 5% normal serum in 1× PBS (pH 7.4). Normal serum was either goat or donkey (depending on the host of the secondary antibody). For monoclonal primary antibodies, the normal serum was replaced with 0.1% bovine serum albumin. Primary antibodies (Table 1) were applied to the sections either for 1 hr at RT or

Abbreviations: CS, chemical sympathectomy; 6-OHDA, 6-hydroxydopamine; DAT, dopamine transporter; TH, tyrosine hydroxylase; VMAT, vesicular monoamine transporter; dopa, dihydroxyphenylalanine.

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for 12 hr at 4°C. After several rinses in PBS, fluorescent secondary antibody was applied for 1 hr at RT in the dark, and the sections were rinsed, coverslipped, and viewed with a Leitz Dialux 20 fluorescent microscope. For double-staining, the above procedure was used, and then the sections were incubated in the secondary primary antibody and processed as described above. The second secondary antibody was conjugated to a different fluorochrome than the first one. For visualizing the TH immunostaining, a tyramide signal amplification system was used (DuPont/NEB) (15). For dopamine immunostaining, strong reducing agents (12) were used in all solutions through the primary antibody incubation to prevent dopamine oxidation. Negative controls included staining with nonimmune rabbit serum, leaving out the primary antibody or the secondary antibody and using several antibodies when possible to recognize the same antigen (Table 1). In the double immunostaining procedures, extra care was taken to avoid any possible cross-reactivity between the different primary and secondary antibodies so that the second secondary antibody did not recognize the first primary antibody. In addition, the double stainings were always repeated reversing the order of the primary antibodies.

### In Situ Hybridization Histochemistry

For all in situ hybridization studies, 200 g male rats were decapitated, the pancreata were removed, rinsed with PBS, and quickly frozen on dry ice. The tissue was kept at −80°C until sectioning. Sections (12 μm thick) were cut in a cryostat (Reichert Frigocut 2500), mounted onto silanized slides, dried on a hotplate at 37°C, and then processed for in situ hybridization histochemistry as described earlier (16). The templates that were used for making the RNA probe are listed below. The sense probe used as a control did not hybridize to the tissue.

Riboprobes were prepared using [35S]UTP and the MaxiScript (Ambion, Austin, TX) kit. The following templates were used. For TH, a fragment of the tyrosine hydroxylase (17) cDNA (a gift of Dona Chikaraishi, Duke University Medical Center, Durham, NC) corresponding to nucleotides 1161–1165 of the coding sequence was used. For dopamine D1b, a fragment of the cDNA between nucleotides 1–1565 (GenBank accession no. M69118) was subcloned into pBluescript KS II+. For transporter mRNAs, specific riboprobes were transcribed from DNA templates generated by PCR using the following primers: for DAT (GenBank accession no. M80570; ref. 18) (nucleotides 1088–1109) TAGAGACGCAATCATCACCA-CC (primer 1, sense) and (nucleotides 1575–1555) CACTGAAATTTGCTGACCGCG (primer 2, anti-sense); for vesicular monoamine transporter 1 (VMAT1) (GenBank accession no. M97380; ref. 19) (nucleotides 1741–1761) AGACAGAGCCAGATGACACAACA (primer 3, sense) and (nucleotides 1986–1971) GCCCTCTAACTGGCAGGAAATGG (primer 4, anti-sense); for VMAT2 (GenBank accession no. L06063; ref. 20) (nucleotides 1649–1669) GACCTCTAATGCTGGAAATGG (primer 5, sense) and (nucleotides 2160–2140) ACACATTTGTACAGTTTACA (primer 6, anti-sense).

For nonradioactive in situ hybridization histochemistry (21), the above riboprobes were labeled with digoxigenin-UTP. After hybridization, the digoxigenin was developed using an anti-digoxigenin antibody conjugated to horseradish peroxidase. The signal then was amplified using a tyramide amplification method (15) and the TSA kit (New England Nuclear). The final marker was fluorescein isothiocyanate-conjugated tyramide.

### Western Blots

Tissues were homogenized in 0.2% Triton X-100 in PBS (pH 7.5), assayed for protein content, and stored at −80°C. Either 20 μg protein of pancreatic tissue or 2 μg protein from adrenal gland was size-fractionated by SDS/PAGE and then electroblotted onto Hybond-Enhanced Chemiluminescence (ECL) nitrocellulose membrane (Amerham) at 30 V for 18 hr in transfer buffer (20% methanol/25 mM Tris/192 mM glycine). Blots were preblocked in 5% nonfat dry milk in Tris-buffered PBS and incubated with anti-TH antibodies for 1 hr at 22°C. Blots were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:1000) for 1 hr at 22°C and developed using the ECL kit (Amerham). Prestained molecular weight markers were used to estimate the molecular weight of immunoreactive species. Antibody dilutions used (also see Table 1) were: Boehringer Mannheim, 1:100 and 1:1000; Incstar, 1:2000; Eugentec, 1:500; Thibault et al. (22) 1:1000.

#### TH Activity Measurements

For determining TH activity, tissues were homogenized in PBS and centrifuged at 20,000 × g for 10 min; the resulting supernatant was recovered. TH activity in the soluble fraction was determined by a modification of the method of Naoi et al. (23) in which NADPH (0.5 mM) and hydroperidine reductase (1 unit per reaction) were used as the reducing system (both from Sigma).

### Southern Blots/PCR

Total RNA (5 μg) from rat pancreas or adrenal was treated with DNase I (amplification grade, Gibco/BRL) for 15 min at 37°C. First-strand cDNA was synthesized using random primers and 200 units of Moloney murine leukemia virus reverse transcriptase (Superscript II, Gibco/BRL) according to manufacturer’s instructions. Subsequently, PCR amplification was performed using 10% of the cDNA in the presence of 0.2 μM of each dNTP, 8 mM MgSO4, 1 μM primers, and 2.5 units of Pwo polymerase (Boehringer Mannheim). Thermocycling conditions were: 1 min at 95°C, 2 min at 55°C, and 2 min at 72°C for 35 cycles followed by 7 min at 72°C. Amplified products were size-fractionated on a 1.4% agarose gel and blotted to nitrocellulose membranes (Schleicher & Schuell). Oligonucleotides were labeled using [γ-32P]ATP and polynucleotide kinase (New England Biolabs), and were hybridized to Southern blots in 3× SSC/2× Denhardt’s solution at 55°C for 18 hr. Final washes were performed in 3× SSC at 55°C.

For amplification of VMAT cDNAs, the following primers were used: For VMAT1, (nucleotides 399–419) CACCTTC-CTGTACCGCGACAGA (primer 7, sense) and (nucleotides 655–635) CTCTCCTAGAATCTATCC (primer 8, anti-sense); for VMAT2, (nucleotides 2161–2181) TTAGGAAATT- TACAATCTCGCA (primer 9, sense) and (nucleotides 2661–2641) GTGAAACTCATTTCTACATTG (primer 10, anti-sense). Oligonucleotides for probing PCR products: For VMAT1, (nucleotides 603–556) GGCTTCATGAGTGAGGGGATGTTGCCATTTCTGGCAAACAGG (oligo 11); for VMAT2, (nucleotides 2300–2347) CATGTTCAACCTTTGAGGTTGACATTTGACACAC (oligo 12).

### RESULTS

Four hours after ligation of the pylorus, the duodenum, and the bile duct, duodenal juice contained a significant amount of dopamine [276 ± 61.8 μg/ml (n = 15)]. Then we used CS to eliminate sympathetic nerve fibers and, thus, rule out neurons
as a source of dopamine. Dopamine content did not change after CS [463 ± 287 pg/ml (n = 5)], suggesting local, non-neuronal production of dopamine.

In the pancreatic tissue itself, 40% of the dopamine content [32 ± 6 (n = 5) versus 84 ± 36 (n = 5) pmol/mg tissue] was still present after CS, in agreement with previously published data (24–26) (Fig. 1). We also measured consistently high levels of DOPA in all samples (data not shown).

The rate-limiting step in dopamine synthesis is the conversion of tyrosine to dopa by TH (27). Therefore, the presence of TH enzyme activity implies the potential for dopamine production. We were able to detect TH activity in the pancreas; the activity was little changed compared with tissue norepinephrine by CS (2.8 ± 0.7 versus 1.8 ± 0.8 pmol/mg protein per min) (Fig. 1).

Using four different antibodies to TH and a signal amplification method (15), we localized TH immunostaining to all exocrine cells of the pancreas (Fig. 2A). The insulin-positive pancreatic islet cells are negative for TH (Fig. 2B). In addition to cells in the exocrine pancreas, numerous nerve fibers were also TH positive, but no neuronal cell bodies were observed. All four anti-TH antibodies gave similar results. To corroborate the histological results, we determined that anti-TH antibodies recognize a protein in pancreatic extracts corresponding in apparent molecular weight to adrenal TH (Fig. 3).

FIG. 1. (Top and Middle) Norepinephrine (NE) and dopamine (DA) content, respectively, of pancreatic tissue. (Bottom) TH activity in the pancreas before and after CS. Note that there is a significant amount of dopamine still present after CS. More than 70% of TH activity also persists after CS. Number of rats in each group: n = 5.

FIG. 2. Immunohistochemical localization of TH, dopamine, and DAT in pancreas. Fluorescent in situ hybridization of VMAT mRNAs. All exocrine pancreatic cells are immunopositive for TH (A), while the insulin-producing islet cells (asterisks) in the same section (B) are negative. (A) An arrowhead points at a nerve fiber. (C) Similarly, all exocrine cells are immunopositive for dopamine itself in addition to sympathetic nerve fibers that are also immunopositive for dopamine (arrowheads). (D) DAT is present in the plasma membrane of all pancreatic cells in addition to the epithelium of excreting ducts (large arrow), veins (small arrows), and some connective tissue elements. (D) Arteries are negative (arrowhead). In situ hybridization histochemistry demonstrates the VMAT1 (E) and VMAT2 (F) mRNAs in all cells (the perinuclear staining is typical of the localization of an mRNA). (Bar = 100 μm.)

Immunostaining for dopamine itself revealed the presence of dopamine in most exocrine cells (Fig. 2C) in addition to sympathetic nerves. We also visualized a few dopamine-positive enterochromaffin cells in the duodenal epithelium. The very scattered distribution and low number of cells make it doubtful that these cells produce sufficient amounts of dopamine to account for the dopamine in the pancreatic/duodenal juice.

Dopamine neurons express a plasma membrane DAT that transports released dopamine back into the cell but that may also function in reverse to release dopamine (for review, see ref. 28). Using an antibody to DAT (13), we found that most exocrine and endocrine pancreatic cells and many vascular elements are DAT immunopositive (Fig. 2D). We consistently observed a plasma membrane immunostaining in the endo-
crine and exocrine cells, in the excreting ducts of the pancreas, and in the venous part of the vasculature. No DAT immunostaining was present in the arterial endothelium (Fig. 2D). Using in situ hybridization histochemistry, we were also able to detect the mRNA encoding DAT in both exocrine and endocrine cells (data not shown).

In neurons and neuroendocrine cells, VMATs package monoamines (serotonin, dopamine, norepinephrine, epinephrine, and histamine) into storage vesicles. Two VMAT subtypes have been identified: (i) VMAT1 (19) from adrenal chromaffin cells and (ii) VMAT2 (19, 20) from monoamine neurons, platelets, and mast cells. Our in situ hybridization histochemistry revealed that both VMAT1 and VMAT2 mRNAs are present in the pancreas (Fig. 2E and F, respectively). To prove the specificity of hybridization, two different riboprobes were used individually with identical results for each transporter. We confirmed the presence of both VMAT1 and VMAT2 mRNA in pancreas by amplification of specific DNA fragments from pancreatic cDNA (Fig. 4). VMAT1 and VMAT2 may be present in different populations of storage vesicles (19, 28).

Finally, we looked for a potential target of the dopamine produced by the pancreas using in situ hybridization histochemistry to visualize mRNAs encoding the five known dopamine receptors. In the pancreas, the dopamine D1b (D5) receptor (29) mRNA is abundantly and widely expressed, whereas the other dopamine receptor mRNAs were not detectable. D1b receptor mRNA was also present in all of the duodenal epithelial cells as was the case for the stomach epithelial cells (unpublished results). Cells in the lamina propria as well as many smooth muscle cells also make a significant amount of D1b receptor mRNA (Fig. 5), in addition to some D3 receptor and D4 receptor mRNAs (data not shown).

DISCUSSION

The pancreas contains markers usually associated with catecholaminergic and neuroendocrine cells. We have shown here in exocrine pancreatic cells the presence of dopamine itself by immunocytochemistry as well as the presence of tyrosine hydroxylase, the enzyme that is responsible for the synthesis of dopamine. Another characteristic of catecholaminergic cells is the presence of transporter molecules. We found that both types of the vesicular monoamine transporters (VMAT1 and VMAT2) are present in the pancreas. The presence of both VMATs in many cells suggests that dopamine, or other monoamines, may be differentially targeted to and costores with specific hormones or enzymes in the pancreas. We have also demonstrated the mRNA encoding the plasma membrane DAT (data not shown) as well as the presence of DAT itself, using immunostaining.

We found both dopamine content and TH activity in the normal and in the CS pancreas and a significant amount of dopamine present in the duodenal juice even after CS. All the sympathetic fibers are destroyed by CS (as indicated by the dramatic decrease in norepinephrine content) (Fig. 1) and, in agreement with literature data (30), we did not detect TH-positive intramural ganglionic neurons in the pancreas. Thus, there is no neuronal source for dopamine production observed after CS. These observations raised the possibility that the pancreas itself may be the major source of dopamine in the duodenal juice.
The immunostaining showed a lack of DAT in the endothelial cells of arteries, while it is rather abundant in excreting ducts and veins. This distribution might be consistent with local production and usage of dopamine. Since the pancreas is a large organ, the dopamine produced here could get into the general circulation and result in high dopamine levels in the blood. This might have harmful cardiovascular effects. One can imagine that the presence of DAT in the veins (and not in the arteries) serves to reuptake the dopamine from the pancreas and metabolize it before it could leak into the general circulation. This suggests that the dopamine made here is intended exclusively for local use.

In looking for a target site of this locally produced dopamine, we demonstrated the presence of mRNA encoding the dopamine D1b receptor in all epithelial cells of the duodenum and the pancreas itself. While the dopamine D1b receptors found in abundance in gastrointestinal epithelial cells seem to be the target of intraluminal dopamine, the pancreatic dopamine receptors might mediate an autocrine feedback effect. The effects of dopamine on exocrine pancreatic secretion have been studied in several animal species (31, 32). While a strong stimulatory effect on secretion was found in dogs (33–35), the effect was less pronounced in other species and is unclear in humans (36).

Our results define a novel catecholaminergic system in the pancreas and duodenum and suggest a paracrine hormonal role for dopamine outside of the central nervous system. Along with acid-secreting parietal cells in the stomach, pancreatic exocrine cells that produce the digestive enzymes synthesize and release dopamine into the duodenum. The pancreatic cells also produce large amounts of dopa, which may serve only as the precursor for dopamine. The possibility that dopa may have a role of its own should be considered, however.

Dopamine appears to have a beneficial effect in acute pancreatitis (37–39). Since we found a similar nonneuronal catecholamine system in the gastric epithelium, it appears that the production and release of dopamine in concert with potentially harmful agents (digestive enzymes) may be a general mechanism of self-defense in the gastrointestinal system. Our results suggest the potential efficacy of specific dopamine receptor agonists in the treatment of gastrointestinal diseases. Future studies should focus on the mechanism whereby dopamine protects the pancreas and duodenum and changes in dopamine secretion in pathological states.

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