Mechanisms in regulating the release of serotonin from the perfused rat stomach

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EVIDENCE HAS ACCUMULATED SHOWING that serotonin regulates gastric motility (26, 30, 31, 35, 42, 44) as well as gastric acid secretion (5, 7, 23, 39) in both in vitro and in vivo models of various animals. In vitro studies showed that serotonin induces contraction of the circular muscle of the guinea pig antrum through 5-hydroxytryptamine (5-HT)2A, 5-HT3 and 5-HT4 receptors (42) and the contraction of the longitudinal muscle of the dog antrum (35). On the other hand, serotonin induces relaxation of the circular muscle of the corpus of the guinea pig through 5-HT1-like receptors (42), and serotonin is involved in the mediation of vagal relaxation of the guinea pig stomach (4). The regulatory role of serotonin on the physiological fed and fasted motor activity of the stomach has been investigated in humans as well as in conscious animal models.

Serotonin initiates gastric phase III of the migrating myoelectric complex (MMC) via 5-HT3 receptors in humans (44). In conscious sheep models, serotonin regulates the MMC of forestomach and antrroduodenal motility through 5-HT4 receptors (30, 31). In conscious guinea pig models, serotonin increases the amplitude of antral contractions through 5-HT3, 5-HT1-like, and/or 5-HT2C receptors (26). Serotonin applied to the serosal side but not the mucosal side produced inhibition of gastric acid secretion from the isolated rat stomach (5). Intracisternal injection of TRH analogs stimulates serotonin release into the gastric lumen in conscious rat models (40), although intraluminal administration of serotonin did not reduce gastric acid secretion. In contrast, systemic administration of serotonin did reduce acid secretion (22). Despite a number of studies that investigated the functional role of serotonin in the stomach, few previous studies have examined the neuronal or hormonal mechanisms that regulate the release of serotonin from the stomach, although it has been extensively investigated in the duodenum and small intestine (14, 17–20).

In the present study, we investigated the effects of intragastric pH and neuronal and hormonal mechanisms on the release of serotonin from the isolated vasoconstrictor and luminal perfused rat stomach. Vascular and luminal release of serotonin were compared, because enterochromaffin (EC) cells in the small intestine are known to release serotonin into the blood circulation as well as into the intestinal lumen, and each direction of the release is mediated by an independent mechanism (14, 15, 25). Intragastric pH seems to be important in regulating the release of serotonin, because serotonin itself plays a role in regulating gastric acid secretion (5, 7, 23, 39). Neuronal regulation of serotonin release is well documented in the duodenum and small intestine, where the cholinergic (10, 15), β-adrenergic (1, 29), and nonadrenergic, noncholinergic (NANC) (14, 34, 36) neuronal pathways are involved.

MATERIALS AND METHODS

Male Wistar rats weighing 200–300 g were used. Care of animals was conducted in accordance with the Guide to the...
Care and Use of Experimental Animals (Shiga University of Medical Science). Animals were housed in a light-controlled room with free access to laboratory food and water but were fasted overnight (16–18 h) before the experiments.

**Immunochemistry for serotonin.** Rats were anesthetized with an injection of pentobarbital sodium (100 mg/kg ip; Nembutal, Abbott Laboratories, North Chicago, IL), perfused for 10 min via the left ventricle with 0.01 M PBS to wash out the blood, and then perfused with the fixative containing 4% paraformaldehyde, 0.5% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer (PB) at 4°C for 10 min at the speed of 30 ml/min. The stomach was taken, immersed in the postfixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M PB at 4°C, and washed for 4 days with several changes of 0.1 M PB containing 15% sucrose. The stomach was cut into 20-μm longitudinal sections in a cryostat and collected in 0.1 M PBS containing 0.3% Triton X-100 (PBST). The sections were incubated with serotonin antibody (13) diluted 1:10,000 in PBST for 3 days at 4°C. To inactivate endogenous peroxidase activity, the sections were incubated at room temperature (RT) for 30 min with 0.1% H2O2 in 0.1 M PBS followed by an additional 20 min with 0.1% phenyl-hydrazine in 0.1 M PBS. After being washed for 30 min with PBST, the sections were incubated at RT for 2 h in biotinylated anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:1,000 in PBST. Sections were then washed and placed in avidin-biotin peroxidase complex (Elite, Vector Laboratories) diluted 1:2,000 in PBST for 2 h at RT. The immunoreactivity was then visualized by incubation with 0.05 M Tris·HCl buffer (pH 7.6) containing 0.01% 3,3′-diaminobenzidine, 1% ammonium nickel sulfate, and 0.0003% H2O2 for 30 min at RT. The stained sections were mounted on gelatin-coated glass slides, dehydrated with graded ethanol, and coverslipped with Entellan (Merck, Darmstadt, Germany). The density of serotonin-positive cells in the epithelium (EC cells) between antrum and corpus was compared. The number of EC cells in the antrum and that in the corpus were separately counted in the longitudinal sections of the stomach, and each value was divided by the corresponding area of the mucosa, which was measured by computer-assisted image analyzing system (27). A mean value of the density of EC cells (cell number/mm²) was determined by sampling from five randomly selected sections, and results were expressed as means ± SD from three animals.

**Vascular and luminal perfusion of the rat stomach.** The animal was anesthetized with an injection of pentobarbital sodium (60 mg/kg ip). Arterial perfusion was achieved through an aortic cannula with the tip lying adjacent to the celiac artery, and effluent perfusate was collected through a portal vein cannula. All vasculature apart from that leading to the stomach was cut between double ligatures. The aortic artery, and effluent perfusate was collected through a cannula placed into the pylorus. The vascular perfusate consisted of Krebs solution containing 3% dextran, 0.2% bovine serum albumin (RIA grade; Sigma Chemical, St. Louis, MO), and 5 mM glucose, and the perfusate was saturated with 95% O2-5% CO2 gas to maintain a pH of 7.4 (11). Luminal perfusates were sequentially changed to 0.1 M HCl (pH 1.0), citrate-phosphate buffer at pH 2, pH 3, pH 5, and pH 7, to examine the effect of different pH on the release of serotonin. Citrate-phosphate buffer at pH 2 was used as a luminal perfusate in other experiments. Both vascular and luminal perfusates and the preparation were kept at 37°C throughout the experiment by a thermostatically controlled heating apparatus. The flow rates for vascular and luminal perfusion were maintained at 3 and 1 ml/min, respectively. After a 25-min equilibration period, both vascular and luminal effluents were collected at 3-min intervals for 33 min into ice-cold vials. Each vial contained 10 μl of 57 mmol/l ascorbic acid, 10 μl of 10 mmol/l EDTA-2Na, 10 μl of 1 mol/l perchloric acid, and 10 μl of 51 mmol/l pargyline hydrochloride in 1 ml of samples (14).

Each of the following chemicals was introduced into the vasculature via a sideward infusion at a final concentration described below for a 12- to 21-min period of perfusion; 1 μM of tetrodotoxin (TTX, Sankyo, Tokyo, Japan), 100 μM of hexamethonium bromide (Sigma Chemical), 1 μM of atropine sulfate (Sigma Chemical), 1 μM of acetylcholine (Sigma Chemical), 0.1 μM of vasoactive intestinal peptide (VIP, human and porcine; Peptide Institute, Osaka, Japan), 1 μM of the VIP receptor antagonist VIP-(10–28) (Sigma Chemical), 1 μM sodium nitroprusside (NaNP), 100 μM of Nω-nitro-L-arginine (L-NNa; Peptide Institute), 0.1 μM of gastrin (human, Peptide Institute), 0.1 μM of somatostatin (Peptide Institute) or 0.1 μM of peptide YY (PYY; Peptide Institute). In some experiments, 1 μM acetylcholine was infused combined with 1 μM atropine; atropine was infused for a 9- to 21-min period, and acetylcholine was infused for a 12- to 21-min period. In some experiments, 0.1 μM somatostatin or 0.1 μM PYY was infused combined with 1 μM TTX; TTX was infused for a 9- to 21-min period, and somatostatin or PYY was infused for a 12- to 21-min period.

The determination of serotonin was performed by HPLC. Vascular effluents were filtrated with Ultrafree-MC (30,000 nominal molecular weight limit, Nihon Millipore, Yonezawa, Japan) by centrifuging for 30 min at 10,000 rpm at 4°C. Luminal effluents were filtrated manually with a 0.22-μm pore disk filter (Millex-GV, Nihon Millipore), 100-μl aliquots of filtrates were injected into HPLC, and serotonin content was measured.

**Statistical analysis.** Results were expressed as means ± SE (in ng/min) in each fraction. Statistical analysis of the data shown in Fig. 2 was performed by use of single-factor ANOVA for repeated measures followed by the Scheffé’s F-test. Percentage of basal release was calculated as 100 × mean serotonin release during drug infusion (12- to 21-min period)/mean basal release (0- to 12- or 0- to 9-min period). A paired t-test (two-tail) was used to compare the values of mean basal release and mean serotonin release during drug infusion in the experiments in Figs. 3–5, and a value of P < 0.05 was considered statistically significant.

**RESULTS**

First we performed an immunohistochemistry of the rat stomach and observed serotonin-immunoreactive cells. Serotonin was positively stained in EC cells and mast cells as well as nerve fibers in the stomach wall (Fig. 1). EC cells are located in the epithelium and extend the cytoplasmic processes toward the lumen, whereas mast cells are located in the lamina propria and are round in shape (Fig. 1). EC cells are found in the antrum and body of the stomach, and the density of EC cells in the antrum (221.9 ± 38.7 cells/mm² of mucosa, n = 3) was significantly (P < 0.01) higher than that in the stomach body (34.7 ± 9.8 cells/mm² of mucosa, n = 3).

The effects of intragastric pH on the release of serotonin into the vasculature as well as into the lumen were examined. At pH 1 and 3, the basal release of serotonin into the vasculature maintained high levels,
but it decreased with the increase in pH (Fig. 2). The release of serotonin into the lumen, on the other hand, was not altered by different pH values in the gastric lumen (Fig. 2). Basal release of serotonin into the vasculature was always higher than that into the lumen at all pH values. In the following experiments, the citrate buffer at pH 2 was always used as a luminal perfusate. In these conditions, basal serotonin release into the vasculature was $10.0 \pm 0.53$ ng/min ($n = 13$) and that into the gastric lumen was $0.87 \pm 0.03$ ng/min ($n = 13$).

To examine the involvement of the cholinergic mechanism in regulating the release of serotonin from the perfused stomach, the effects of TTX, hexamethionium, and atropine were examined. TTX and hexamethionium significantly reduced the basal vascular serotonin release, whereas atropine did not affect the basal vascular release of serotonin (Fig. 3). Infusion of acetylcholine significantly reduced the basal vascular release of serotonin, and this inhibitory effect was completely antagonized by atropine (Fig. 3). In these experiments, basal release of serotonin into the lumen was affected by neither of these chemicals (data not shown).

The involvement of inhibitory neurotransmitters such as VIP and nitric oxide (NO) on the serotonin release was examined. Neither VIP nor NO donor NaNP affected the basal vascular release of serotonin (Fig. 4). Furthermore, VIP(10–28) and the NO biosynthesis inhibitor L-NNA did not affect the basal vascular release of serotonin (Fig. 4). Again, the basal release of serotonin into the lumen was not affected by either of these chemicals (data not shown).

The effects of hormones on the serotonin release were examined. Gastrin exerted no effects; however, somatostatin and PYY significantly reduced the basal

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**Fig. 1.** Immunohistochemistry for serotonin in the corpus (A) and antrum (B, C) of the rat stomach. Serotonin-positive cells are densely packed in the antral mucosa (B) but sparsely distributed in the corpus (A). In a more highly magnified view of the antrum (C), enterochromaffin (EC) cells (small arrows) in the epithelium, mast cells (large arrows) in the lamina propria, and nerve fibers (arrowheads) in the submucosa are positively stained. Bars = 100 μm (A, B) and 50 μm (C).

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**Fig. 2.** Effects of different intragastric pH on the vascular and luminal release of serotonin from the perfused rat stomach. Vascular release of serotonin maintains higher levels at pH 1 and 3, but it decreases with increases in pH. Luminal release of serotonin is significantly lower than vascular release at all pH values and is not altered by different pH values. Values represent means ± SE from 6 animals; *$P < 0.05$, **$P < 0.01$ compared with the vascular release at pH 1.
vascular release of serotonin (Fig. 5). These inhibitory effects exerted by somatostatin and PYY were not altered by the combined administration with TTX (Fig. 5). The basal release of serotonin into the lumen was not affected by either of these chemicals (data not shown).

DISCUSSION

Immunohistochemistry for serotonin revealed that EC cells were numerous in the antrum, sparsely scattered in the corpus, and not found in the fundus of the rat stomach. Although serotonin is also contained in the mucosal mast cells and enteric neurons (13) of the rat stomach, the serotonin content of the EC cells is much higher than that of mast cells or neurons. Therefore, serotonin detected in the present study most likely originates from the EC cells, although one cannot rule out the possibility that it originates from the mast cells or enteric neurons, whose cell bodies are located in the myenteric plexus and extend fibers throughout the stomach wall (13).

In the present study, we perfused the rat stomach vascularly and luminally, and the release of serotonin into the vasculature and into the gastric lumen was compared. In experimental conditions using pH 2 as a luminal perfusate, basal release of serotonin into the vasculature was more than 10 times higher than luminal serotonin release, and, moreover, only vascular release of serotonin was affected by drug treatments, whereas luminal release of serotonin was hardly affected. These results were consistent with the previous report, which showed that the basal release of serotonin into the portal circulation was 1,000 times higher than the luminal serotonin release in the anesthetized rat stomach (22). Previous immunoelectron microscopic study combined with biochemical measurements of serotonin release has shown that serotonin detected in the lumen is released from the apical site of the EC cells, whereas serotonin detected in the vasculature is released from the basolateral site of the EC cells (12). The present and previous results suggest that serotonin is mainly released from the basolateral site of the EC cells but hardly released from the apical site of the EC cells in the stomach. Serotonin released from the basolateral site of the EC cells may reach the connective tissue space of the lamina propria and exert paracrine effects on either endocrine cells such as D, G, or ECL cells (21, 22) or intrinsic and extrinsic nerve terminals (8, 28, 35, 37, 42, 44). The local action of

Fig. 3. Effects of tetrodotoxin (TTX), hexamethonium (C6), atropine (ATR), acetylcholine (ACh), and ACh + ATR on vascular release of serotonin from the perfused rat stomach. Percentage of basal release is calculated as 100 × serotonin release during drug infusion/basal serotonin release. Values are means ± SE; no. of rats is shown at base of bars. *P < 0.05, **P < 0.01 compared with basal release.

Fig. 4. Effects of vasoactive intestinal peptide (VIP), the VIP receptor antagonist VIP-(10–28), sodium nitroprusside (NaNP), and N(G)-nitro-L-arginine (L-NNA) on vascular release of serotonin from the perfused rat stomach. Values are means ± SE; no. of rats is shown at base of bars.

Fig. 5. Effects of gastrin, somatostatin (Soma), peptide YY (PYY), soma + TTX, and PYY + TTX on vascular release of serotonin from the perfused rat stomach. Values are means ± SE; no. of rats is shown at base of bars. *P < 0.05, **P < 0.01 compared with basal release.
serotonin in the lamina propria is terminated by the serotonin transporter-mediated uptake by epithelial cells to minimize its spread in the tissue or into the lumen (6, 43). Vectoral release of serotonin has been examined in the rat duodenum, where the basal release of serotonin into the lumen is higher than that into the vasculature, and luminal release is always affected by drug treatments, whereas vascular release is hardly affected (14). Therefore the direction of serotonin release and its response to drug treatment in the stomach were quite different from those in the small intestine, where serotonin released from apical site of the EC cells may regulate the epithelial secretion or ion transport (2, 9). Electron microscopic studies have demonstrated that secretory granules in the EC cells in the antrum are only distributed in the basal cytoplasm (38), whereas those in the duodenum are located in both apical and basal cytoplasm (12); such morphological differences in the distribution of serotonin-containing granules in the EC cells may explain the difference in the direction of serotonin release.

We examined the effects of different pH values in the gastric lumen on the release of serotonin from the perfused stomach. Results showed that luminal acidification stimulated the vascular release of serotonin but did not affect the luminal release of serotonin. Similar results have been shown in the rabbit duodenum, where luminal acidification stimulates the release of serotonin into the lumen as well as into the vasculature (17–20). It is well known that serotonin inhibits acid secretion in the stomach (5, 7, 22, 23, 39). It has been reported that exogenously applied serotonin inhibits acid secretion, and this effect is exerted by serotonin applied into blood vessels but not by serotonin applied into the gastric lumen (22). Judging from both previous and present results, serotonin released from the basolateral site of the EC cells seems to be important in the regulation of gastric acid secretion, but serotonin released from the apical site of the EC cells is of less importance. Basolaterally released serotonin may exert paracrine effects on the parietal cells or indirectly on gastric mucosal enteroendocrine cells such as ECL, D, or G cells (21) to regulate the acid secretion.

Results showed that TTX and hexamethonium reduced the vascular release of serotonin, although atropine had no effect. These results suggest that the release of serotonin from the stomach is controlled by neuronal pathways: the cholinergic nicotinic pathway seems to be involved, but the cholinergic muscarinic pathway does not appear to be directly involved. Direct effects of TTX on serotonin release by the blockade of voltage-activated sodium channels on the EC cells cannot be denied (32, 33). Sodium-dependent depolarization via voltage-activated sodium channels followed by calcium influx via voltage-activated calcium channels has been shown to be important in the release of serotonin from the EC cells (33). It has been shown that the blockade of voltage-activated sodium channels induced 30% reduction of the serotonin release, whereas the blockade of voltage-activated calcium channels induced 70% reduction of the serotonin release (33). The reduction of the serotonin release by TTX shown in the present study might be due not only to direct blockade of voltage-activated sodium channels on the EC cells but also to indirect blockade of neuronal input to the EC cells. Since the reduction of serotonin release induced by TTX or hexamethonium is only slight, other inhibitory mechanisms such as the endocrine mechanism seem to be involved in regulating the release of serotonin. Acetylcholine reduced serotonin release, and this effect was antagonized by atropine, but atropine itself did not affect serotonin release. These results suggest that acetylcholine indirectly affects serotonin release via a muscarinic receptor, which may be located on the endocrine cells or inhibitory neurons. Both hexamethonium and acetylcholine reduced serotonin release; this can be explained by the fact that the release of serotonin stimulated via nicotinic receptors may reach to the submaximal level in the condition of intraluminal pH 2, and therefore inhibitory mechanisms via muscarinic receptors may be more sensitive to acetylcholine than stimulatory mechanisms via nicotinic receptors.

The effects of VIP and NO on the gastric functions have been widely studied, although most of the previous studies examined the effects on gastric motility (16, 24, 41) and only a few examined the effects on the release of serotonin. The present results showed that neither VIP, VIP-(10–28), NaNP, nor L-NNA affected serotonin release. Because the basal release of serotonin is tonically stimulated by the luminal acidification as well as the cholinergic nicotinic pathways as mentioned above, negative response of the basal release to VIP, VIP-(10–28), NaNP, or L-NNA affected serotonin release. Because the basal release of serotonin is tonically stimulated by the luminal acidification as well as the cholinergic nicotinic pathways as mentioned above, negative response of the basal release to VIP, VIP-(10–28), NaNP, or L-NNA affected serotonin release. Because the basal release of serotonin is tonically stimulated by the luminal acidification as well as the cholinergic nicotinic pathways as mentioned above, negative response of the basal release to VIP, VIP-(10–28), NaNP, or L-NNA affected serotonin release. Because the basal release of serotonin is tonically stimulated by the luminal acidification as well as the cholinergic nicotinic pathways as mentioned above, negative response of the basal release to VIP, VIP-(10–28), NaNP, or L-NNA affected serotonin release. Because the basal release of serotonin is tonically stimulated by the luminal acidification as well as the cholinergic nicotinic pathways as mentioned above, negative response of the basal release to VIP, VIP-(10–28), NaNP, or L-NNA affected serotonin release. Because the basal release of serotonin is tonically stimulated by the luminal acidification as well as the cholinergic nicotinic pathways as mentioned above, negative response of the basal release to VIP, VIP-(10–28), NaNP, or L-NNA affected serotonin release. Because the basal release of serotonin is tonically stimulated by the luminal acidification as well as the cholinergic nicotinic pathways as mentioned above, negative response of the basal release to VIP, VIP-(10–28), NaNP, or L-NNA affected serotonin release.

Effects of endocrine mechanisms on the release of serotonin from the stomach were examined. Somatostatin reduced vascular release (decreased to 60% of the basal level), as did PYY (decreased to 40% of the basal level), but gastrin had no effect. The inhibitory effects of somatostatin and PYY were not antagonized by TTX. These results suggest that somatostatin and PYY may exert direct inhibitory effects on serotonin release without the mediation of neuronal pathways. The localization of somatostatin receptors on the EC cells has been reported previously (32); therefore, endogenously released somatostatin, not merely that applied exogenously, may act directly on the EC cells. Somatostatin-containing D cells are located in the epithelium of the antrum and corpus of the stomach, whereas PYY-containing cells are located in the epithelium of the distal small intestine and colon. Therefore PYY may exert blood-borne effects and somatostatin may exert paracrine rather than endocrine effects, possibly via somatostatin and PYY receptors located on the EC cells (14).
the EC cells. Because it has been shown that cholinergic muscarinic receptors are located on antral D cells (3), the cholinergic muscarinic pathways may act indirectly on EC cells through somatostatin-containing D cells in the stomach. This might be a partial explanation for the inhibitory effect of acetylcholine on the release of serotonin.

In conclusion, the present study showed that serotoninin-containing EC cells were densely distributed in the antrum, and these cells release more serotonin into the vasculature than into the gastric lumen. Vascular release of serotonin was increased at low pH levels of gastric lumen but decreased at higher pH levels. The vascular release of serotonin was regulated by cholinergic nicotinic mechanisms but not by VIP and NO pathways. Somatostatin and PYY originating from endocrine cells appeared to exert direct inhibitory effects, possibly via somatostatin and PYY receptors on EC cells, and the cholinergic muscarinic mechanism might exert indirect effects on serotonin release, possibly via muscarinic receptors on somatostatin-containing D cells.

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