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Immunohistochemical Demonstration of $GABA_B$ Receptors in the Rat Gastrointestinal Tract*

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Immunohistochemical localization of $GABA_B$-receptors was demonstrated in the rat gastrointestinal tract using a monoclonal antibody (GB-1) raised against the purified $GABA_B$-receptor. Immunoreactive staining for $GABA_B$-receptors was found in some populations of endocrine, muscular and neuronal components in the stomach and gut wall. Positive mucosal epithelial, probably endocrine, cells were distributed throughout the stomach and intestine. Double immunostaining indicated that such positive cells for $GABA_B$-receptors often co-possessed serotonin in the small intestine but not in the gastric body. In the muscular layer of the digestive canal, positive staining was seen as dotty granules punctuated on the surface of muscle fibers. In the enteric nervous system, positive neuronal somata were found in both submucosal and myenteric ganglia throughout the entire canal extending from the stomach to the rectum. This is the first report to visualize the cellular localization of $GABA_B$-receptors in the gastrointestinal system of the rat, and should provide a fundamental basis for future studies on gastrointestinal functions regulated by $GABA_B$-receptors.

KEY WORDS: $GABA_B$-receptor; immunohistochemistry; gastrointestinal tract; rat.

INTRODUCTION

The inhibitory amino acid neurotransmitter γ-aminobutyric acid (GABA) has been known to play important roles in both central and peripheral nervous systems (reviewed by Ong and Kerr in 1). In peripheral digestive organs, moreover, GABA has been detected not only in the enteric nervous system (2–4) but also in endocrine cells (5,6). Thus, GABA has been thought to act as a multi-functional molecule in the gastrointestinal system, probably exerted by different types of GABA receptors.

There is increasing pharmacological evidence that GABA receptors are classified into two subtypes; $GABA_A$ (ionotropic) and $GABA_B$ (metabotropic) (7, reviewed by Kuriyama in 8). The distinguishable moduratory effects of these receptors have been reported in the gastrointestinal tract (9-13). For example, the vascular infusion of GABA to vascularly perfused rat stomach induced a dose-dependent increase in gastrin release and this effect of GABA was unaffected by the $GABA_B$ antagonist delta-aminovaleic acid, but was fully prevented by $GABA_A$ antagonist bicuculline (12). Contraction caused by GABA in the guinea-pig ileum was antagonized by bicuculline (9,10) and was mimicked by $GABA_A$ agonist muscimol (10). Baclofen, a $GABA_B$-receptor agonist, however reduced the intestinal contractions (9-11, 13). These observations strongly suggest that different subtypes of GABA receptors may be responsible for different actions of GABA in the gastrointestinal system. To reply this question precisely, it is essential to clarify the anatomical distribution of each subtype of GABA receptors at a cellular level.

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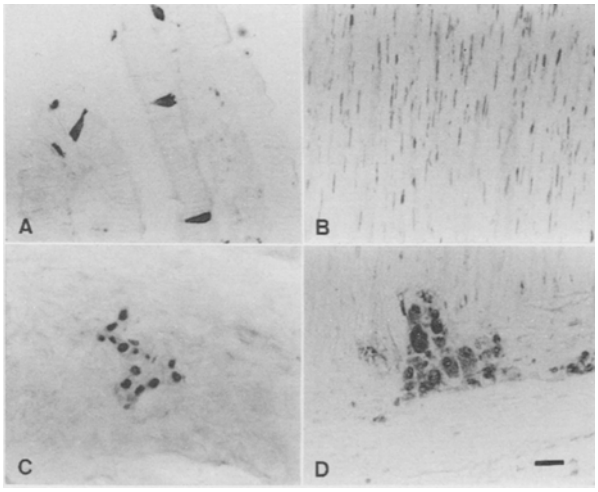

Fig. 1. Immunohistochemistry for $GABA_B$-receptors using GB-1 antibody. (A): Duodenal mucosa. Some positive mucosal cells display thin processes extending toward the lumen. (B): Circular muscle layer of the duodenum. Positive staining is seen as dotty puncta. (C): Duodenal submucosal ganglion. Nuclei of ganglionic cells are labeled intensely. (D): Colonic myenteric ganglion. Cell bodies and their nuclei are labeled. Bar = 25 μm.

Although the distribution pattern of $GABA_A$-receptors in the rat stomach has been studied by autoradiography (14), little is yet known about the distribution of $GABA_B$-receptors. In the present study, we first demonstrated the immunohistochemical localization of possible $GABA_B$-receptors by using a monoclonal antibody (GB-1) raised recently against $GABA_B$-receptors (15).

EXPERIMENTAL PROCEDURE

Tissue Preparation. Ten male Wistar rats (Clea Japan Inc., Japan) weighing 250-300 g were used. Under pentobarbital anesthesia (50 mg/kg), the animals were perfused via the ascending aorta with 10 mM phosphate buffer containing 0.9% NaCl (pH 7.4) followed by a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The rats were kept in crushed ice during the perfusion. After the perfusion, tissue specimens taken from the stomach or the gut including the rectum were quickly dissected out, cut longitudinally by scissors, and flattened on a cork plate with pins. The specimens were immersed and kept for 1 day in the same fixative. After cryoprotection by placing for at least 3 days in 0.1 M phosphate buffer containing 15% sucrose, the tissues were frozen with dry-ice and cut into 20-μm-thick sections. The sections were collected in 0.1 M phosphate-buffered saline, pH 7.4, containing 0.3% Triton X-100 (PBST). All the above procedures were carried out at 4 °C.

Immunohistochemistry. The sections in a free-floating state were first incubated for 5 to 7 days with a primary monoclonal antibody (GB-1; diluted 1:150,000, 0.13 μg IgM/ml) at 4 °C. After washing with PBST, sections were treated with biotinylated secondary antibody (Vector Laboratory, Burlingame, CA; 1:1,000) for 1 hour at room temperature, followed by incubation in the avidin-biotin-horseradish peroxidase (ABC Elite, Vector Laboratory, Burlingame, CA; 1:4,000) for 1 hour at room temperature. Dilution of antisera and washing of sections were all done with PBST. A dark blue or purple was developed by reacting the sections for 20 min with a reaction mixture containing 0.01% 3,3'-diaminobenzidine (DAB), 0.0045% H_2O_2 and 0.3% nickel ammonium sulfate in 50 mM Tris-HCl buffer (pH 7.6). The stained sections were mounted on gelatin-coated glass slides, air-dried, dehydrated and cover-slipped. Some sections were counter-stained with nuclear fast red for histological examination.

Production and characterization of the monoclonal antibody, GB-1, have been reported previously (15,16). In brief, the GB-1 antibody was raised against a partially purified preparation for $GABA_B$-receptors. This antibody recognized a protein of about 80 kDa contained in a soluble synaptic membrane preparation of the bovine brain. Immunoabsorbent agarose beads conjugated with the antibody were able to remove $GABA_B$-receptor binding activity from the synaptic membrane fraction. Immunoaffinity purification and characterization have provided good evidence that the 80 kDa antigenic molecule represents a $GABA_B$ receptor protein.

Double Immunostaining for GB-1 and Serotonin or Somatostatin. After completion of the first DAB reaction for GB-1 staining, sections were treated with 0.5% H_2O_2 in PBST for 30 min to kill any residual peroxidase from the first cycle. Sections were then incubated with a rabbit polyclonal antibody against serotonin (diluted 1:40,000) (17) or somatostatin (diluted 1:10,000; Immuno Nuclear Corp.) for 5 days at 4°C. Second cycle immunostaining utilized the standard DAB method to give a brown precipitate, which contrasted well with the dark purple reaction products of the first cycle staining.

Double Staining for GB-1 and NADPH-Diaphorase. For double staining by GB-1 immunohistochemistry and NADPH-diaphorase histochemistry, the sections were first stained for NADPH-diaphorase histochemistry according to the method reported previously (18). In brief, NADPH-diaphorase activity was rendered visible by incubating the sections for 2 hours in a reaction mixture containing 0.01 mM NADPH (Kojin Co., Japan), 0.02 mM nitro blue terazolium (Sigma, USA) and 0.3% Triton-X in 0.1M phosphate-buffered saline (pH 8.0) at 37°C. The reaction was terminated by washing the sections in 0.1 M phosphate-buffered saline. Blue-stained sections were then immunostained using GB-1 (diluted 1:150,000) according to the standard DAB method as described above.

RESULTS

Immunoreactive staining for $GABA_B$-receptors was observed in some populations of epithelial (Fig. 1A), muscular (Fig. 1B) and neuronal (Figs. 1C and 1D) components, as exemplified in the duodenum and colon. In the muscular layer, positive staining for $GABA_B$-receptors is seen as dotty puncta deposited in adjacent to the surface of muscle fibers (Fig. 1B). Such positive puncta were more abundant in the circular muscle layer than in the longitudinal muscle layer (data not shown).

In the enteric nervous system, neuronal somata positively stained for $GABA_B$-receptors were observed in both submucosal (Fig. 1C) and myenteric plexuses (Fig. 1D) of the entire digestive canal examined from the stomach to the rectum. Although positive reaction prod-

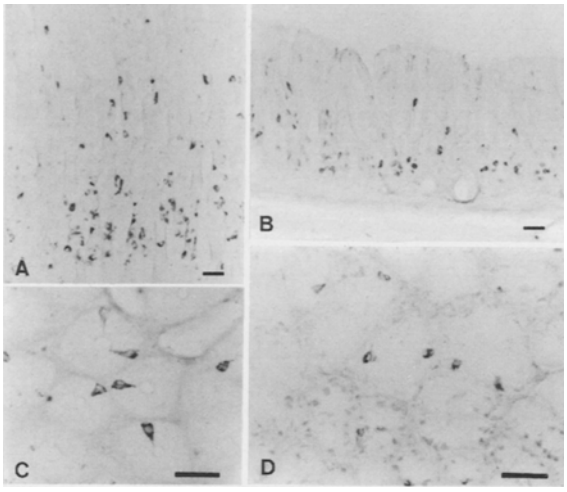

Fig. 2. Structures positive for $GABA_B$-receptors in transverse sections of the mucosal epithelium of the gastric body (A), antrum (B), duodenum (C) and colon (D). Note that positive cells are distributed mainly in the basal layer of the stomach. Bars = 50 μm.

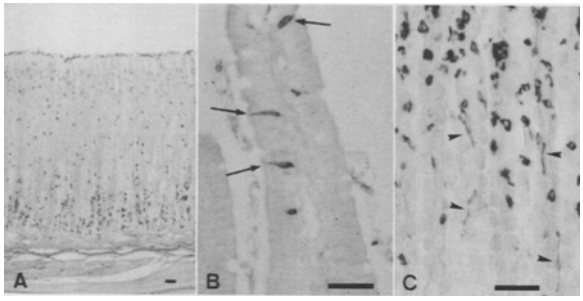

Fig. 3. Double immunostaining for $GABA_B$-receptors and serotonin (A and B) or somatostatin (C). Immunoreactivity for $GABA_B$-receptors is stained by deep purple, while serotonin or somatostatin is labeled by brown. In the gastric body (A), immunoreactive cells for $GABA_B$-receptors (purple) are distributed mainly in the basal layer, whereas serotonin positive cells (brown) are seen in the upper layer. In the duodenal mucosa (B), positive cells for $GABA_B$-receptors contain serotonin (arrows). In the gastric body (C), somatostatin positive cells indicated by brown are not positive for $GABA_B$-receptors (arrowheads). Bars = 50 μm.

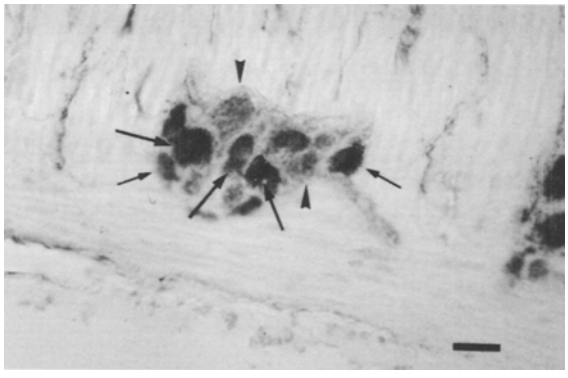

Fig. 4. Double staining for NADPH-diaphorase histochemistry (blue) and GB-1 immunohistochemistry (brown) in the duodenal myenteric ganglion. Arrows indicate doubly labeled cell, and arrowheads indicate cells singly positive for GB-1. Bar = 50 μm.

ucts with a moderate staining intensity were present at the cell membrane and cytoplasm of ganglionic cells, the nucleus was sometimes stained much intensely (Fig. 1C).

In the mucosal epithelium (Fig. 2), positive cells, often extending single thin processes toward the lumen to imply endocrine in their nature, were intermingled with unstained epithelial cells. These positive cells were found throughout the digestive canal extending from the stomach to the rectum. However, the density of positive cells varied from region to region. In the gastric body (Fig. 2A) and antrum (Fig. 2B), for example, many positive cells were seen in the mucosal epithelium, tending to be situated in the basal layer of the gastric gland. In the small intestine (Fig. 2C), on the other hand, positive epithelial cells with a low density were detected at the upper layer of the mucosal layer. Caudally, the density of positive epithelial cells was further decreased gradually, and only a few positive cells were recognized in the colon (Fig. 2D).

Figures 3A and 3B show double immunostaining for $GABA_B$-receptors and serotonin in the epithelial mucosa of the stomach and duodenum, respectively. In the gastric body (Fig. 3A), positive cells for $GABA_B$-receptors are virtually devoid of positive staining for serotonin. As noted above, positive cells for $GABA_B$-receptors are distributed mainly in the basal layer, while serotonin-positive cells are mostly located in the upper layer. In the duodenum, however, positive cells for $GABA_B$-receptors almost always revealed serotonin immunoreactivity (Fig. 3B). Fig. 3C shows double immunostaining for $GABA_B$-receptors and somatostatin in the gastric body. It is clearly understood that somatostatin-positive cells are not positive for $GABA_B$-receptors.

Fig. 4 shows a typical example of double staining of immunohistochemistry for $GABA_B$-receptors and enzyme histochemistry for NADPH-diaphorase in the duodenal myenteric plexus. The large majority of the ganglionic neurons appeared to stain positively for $GABA_B$-receptors, and about a half of such positive cells exhibited NADPH-diaphorase activity.

DISCUSSION

The monoclonal antibody, GB-1, was raised against a partially purified preparation for $GABA_B$-receptors

(15). Although the $GABA_B$ receptor has not been cloned, the pharmacological property of the purified preparation was established by several approaches. The preparation mimicked the $GABA_B$-receptor in its binding activity for GABA, baclofen (a $GABA_B$ receptor agonist) and 2-hydroxysaclofen (a $GABA_B$ receptor antagonist). Immunoabsorbent agarose beads conjugated with the antibody were able to remove the baclofen-suppressive GABA-binding activity in a soluble synaptic membrane fraction of the bovine brain. In the bovine brain homogenate, the GB-1 antibody recognized an 80 kDa protein. When the protein was reconstituted with GTP-binding protein and an adenylylcylase preparation on a phospholipid membrane, the adenylylcyclase activity was inhibited by the addition of GABA or baclofen. These results all indicate that the monoclonal GB-1 antibody recognizes a $GABA_B$-receptor (16). In the present study, therefore, positive staining obtained with the GB-1 antibody can be assumed to represent the site of $GABA_B$-receptors.

In the gastrointestinal mucosa, some epithelial cells were strongly labeled with the GB-1 antibody. Previous studies have shown that GABA-containing enteric ganglionic cells had little projection to the mucosal layer (3,4). However, a subpopulation of mucosal epithelial cells have been shown to contain GABA in the rat stomach (19). Therefore, it is likely that GABA released from these GABA-containing mucosal cells may act in a paracrine fashion on mucosal epithelial cells which bear receptors of either $GABA_A$ or $GABA_B$ or both. Indeed, the presence of $GABA_A$ receptors, at least in basal parts of the gastric gland, has been proposed (14). Since the morphological feature of $GABA_B$ receptor-positive mucosal cells generally resemble the characteristic shape of endocrine cells, it is possible to imagine that GABA is utilized as a local hormone between endocrine cells within the gastrointestinal mucosa. Such an assumption was demonstrated, at least partly in the duodenum studied here, by the fact that mucosal epithelial cells expressing $GABA_B$-receptors do contain an endocrine monoamine serotonin. This finding agrees well with the pharmacological study demonstrating that the release of serotonin from intestinal enterochromaffin cells was directly inhibited by $GABA_B$-receptors (20). It should be noted, however, that gastric mucosal cells stained positively for $GABA_B$-receptors did not contain serotonin. Thus, the regulatory role of GABA on serotonin secretion in the duodenum may differ from that in the stomach.

In the muscular layer, particularly of the small intestine including the duodenum, positive staining for $GABA_B$ receptor was seen as dotty staining mainly in the circular muscle. This result coincides with an autoradiographic study using [^{3}H]GABA (21), showing that dense clusters of silver grains occurred over the circular muscle. It has been further suggested that GABAergic innervation to the circular muscle may indicate the prejunctional modulation of transmitter release from neighboring axons (22). Our data raise a possibility that GABA released from GABAergic terminals acts via $GABA_B$-receptors expressed on their own or other nerve terminals innervating the circular muscle. To clarify this issue, immunoelectron microscopic examination should be required in future studies.

In the enteric nervous system, many neuronal somata in both submucosal and myenteric plexuses were positive for $GABA_B$-receptors. In agreement with this observation, a previous immunohistochemical study using GABA antiserum reported that myenteric ganglionic cells were surrounded by GABA-containing nerve fibers and terminals (3). It has been also suggested that ganglionated plexuses of the intestine possessed very few $GABA_A$ receptors (14). Taken altogether, it is clearly indicated that intramural ganglionic cells of the gastrointestinal organ may receive GABAergic control mediated by $GABA_B$ receptors. $GABA_B$ receptor-mediated inhibition on intestinal motility have been repeatedly described on the basis of in vitro studies (9–11, 13). Although an assessment is required for studies with systemic injection of baclofen, the effects may include central mechanisms because the drug easily penetrate the blood-brain-barrier. In any event, the $GABA_B$ receptors proved here to be present in ganglionic cells may relate with the relaxation of the stomach and gut. In this context, it may be worthy to note that nitric oxide is also a potent relaxant of gastrointestinal movement (23). Interesting enough, the present study has revealed that the enteric neurons immunoreactive for GB-1 are partly positive for NADPH-diaphorase which is a histochemical marker of neuronal nitric oxide synthese (24). This result strongly suggests the intimate and harmonious relationship between $GABA_B$ receptors and nitric oxide in gastrointestinal relaxation. It should be kept in mind, however, there are many other enteric neurons that are positive for GB-1 but negative for NADPH-diaphorase activity. At present, it is uncertain which neurotransmitter(s) these GB-1-positive neurons utilize.

In conclusion, the present study first demonstrates the cellular localization of $GABA_B$-receptors in the rat digestive tract. Various functions of the gastrointestinal system appear to be controlled via $GABA_B$-receptors expressed in not only neuronal but also endocrine cells.

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