Anatomical and Neuropeptidergic Properties of the Duodenal Neurons Projecting to the Gallbladder in the Golden Hamster

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Summary. This study investigated the anatomical and neuropeptidergic properties of the duodenal neurons projecting to the gallbladder in the golden hamster. Fast blue (FB) was injected into the subserosa of the gallbladder in order to identify by retrograde tracing the duodenal neurons that project to the gallbladder. Subsequently, immunofluorescence microscopy was employed to see whether these duodenal neurons contained putative peptidergic neurotransmitters such as calcitonin gene-related peptide (CGRP), galanin (GAL) and vasoactive intestinal polypeptide (VIP).

The FB-labeled cells were only found in the duodenal region adjacent to the major duodenal papilla where the biliary duct opens. On the other hand, there was no difference within this duodenal region in the numbers of FB-labeled cells between the mesenteric and antimesenteric portions, suggesting that these two portions of the duodenum equally contribute neuronal projections to the gallbladder. Double-immunofluorescence microscopy clearly demonstrated that a small population of FB-positive duodenal neurons contained putative neurotransmitters CGRP, GAL and VIP.

Our data suggest that duodenal neurons around the major duodenal papilla in the golden hamster project to the gallbladder and exert their influence on the gallbladder via neuropeptides such as CGRP, GAL and VIP.

Numerous physiological studies indicate that the primary effects of the intestine on the gallbladder are mediated by hormones, notably cholecystokinin (CCK), together with other hormones such as pancreatic polypeptide, gastrin and secretin (Eyssselein et al., 1983; Nealon et al., 1990; Liddle, 1995). The gallbladder also contains an extensive ganglionic plexus and possesses nerve fibers in close proximity to its muscle and epithelium (Sand et al., 1993; Talmage et al., 1996). This ganglionated plexus may contain circuits for intrinsic gallbladder reflexes, and it could serve as an integrating center for extrinsic vagal and sympathetic effects (Yau and Youther, 1984; Dahlstrand, 1990), in addition to being involved in intestino-biliary co-ordination. The last possibility is supported by anatomical studies that have demonstrated direct neural pathways from the duodenum to the gallbladder in both the guinea pig and the Australian possum (Mawe and Gershon, 1989; Padbury et al., 1993). The hamster, unlike the rat and mouse, has a gallbladder and is often used in physiological studies such as hibernation (Galluser et al., 1988). The anatomical properties of calcitonin gene-related peptide (CGRP), galanin (GAL) and vasoactive intestinal polypeptide (VIP) in the gallbladder have been extensively examined in numerous animals, since the direct effect of these neuropeptides on the motility of the gallbladder is relatively well understood (Sundler et al., 1977; Ryan and Ryan, 1978; Goehler et al., 1988; Harling et al., 1991; Sand et al., 1993; El-Salhy et al., 1996; Talmage et al., 1996; Rasmussen et al., 1997). However, little is known about neuropeptidergic properties of duodenal neurons projecting to the gallbladder.

In the present study, we have demonstrated the
Fig. 1. Micrographs of retrogradely labeled nerve cell bodies of myenteric ganglia in the mesenteric region (A) and anti-mesenteric region (B). Two or three labeled cells were found mainly within a ganglion; there was no difference in the frequencies of fast blue-labeled cells between the mesenteric region and anti-mesenteric region. Scale bar = 50 μm

presence as well as the neuromodulatory properties of duodenal neurons projecting to the gallbladder of the hamster.

MATERIALS AND METHODS

Fourteen hamsters (120-150 g body weight) of both sexes were used in this study. All animals were fasted for 12 h before use. The hamsters were anesthetized with pentotal sodium (thiopentone sodium; Choon-gwae Pharma Co., Korea) and a midline abdominal incision was made to expose the gallbladder. A beveled glass micropipette was used to inject the wall of the gallbladder with a total of 1-3 μl of fast blue (4% in distilled water; Sigma, USA) in 2 or 3 sites (n=10). As controls, the tracer dye was placed into the peritoneal cavity (n=2) and on the peritoneal surface of the gallbladder (n=2) so as to evaluate a non-specific labeling that might be produced in duodenal neurons by the direct spread of the tracer.

Two days after the injection of fast blue (FB), 5 mg/kg colchicine (Sigma, USA) dissolved in 1 ml of saline was injected intraperitoneally (i.p.) into the animals 18 h before death to increase the concentration of neuropeptides in the nerve cell bodies, as described in a previous study (Furness et al., 1984). The animals were re-anesthetized, and perfused via the ascending aorta with 200 ml of 4% paraformaldehyde in a phosphate buffer (PB; 0.1 M, pH 7.2). The animals used in this experiment were treated in accordance with the 'Principles of Laboratory Animal Care' (NIH publication No. 85-23, revised 1985).

A 4 cm segment of the duodenum around the major duodenal papilla was removed from each animal, cut open, and pinned on cork plate and postfixed in the same fixative for 4 h. The tissue was cryoprotected by serial sucrose infiltrations, embedded in OCT compound, and frozen rapidly. Tissue specimens were cut into 12 μm coronal and longitudinal sections on a cryostat (Reichert Jung, Germany).

The sections were incubated overnight at room temperature in rabbit anti-rat CGRP, anti-rat GAL and anti-rat VIP sera (Peninsula, USA), respectively diluted to 1:2500, 1:1000, and 1:5000 in phosphate buffered saline (PBS) containing 0.3% Triton X-100 and 2% normal goat serum, and then further incubated in Cy3 conjugated goat anti-rabbit IgG (Jackson Immunochemicals, USA), diluted 1:300 in the same diluent for the primary antiserum. The sections were mounted on a gelatin-coated slide in Crystal mount (Biomed, USA), and observed under an Axiosplan fluorescence microscope (Carl Zeiss, Germany). The same sections were stained with cresyl violet after the cover glasses were removed. As an immunofluorescence control, each antiserum was pre-absorbed with 100 μg/ml of synthetic CGRP, GAL and VIP (Sigma), which caused an almost complete disappearance of immunoreactivity in all areas investigated.

To compare the frequency of FB-labeled cells between the mesenteric and anti-mesenteric portions of the duodenum, the number of FB-labeled cells in the mesenteric and anti-mesenteric half of the duodenum were counted and the frequency was calculated by expressing the number of FB-labeled cells as a percentage of total neurons identified by cresyl violet staining. Student’s t- and paired t-tests were used for statistical analysis.

RESULTS

In eight out of ten injected animals, FB-labeled nerve cell bodies were observed in the myenteric plexuses of the duodenum. They were confined to a 20 mm segment of the duodenum that extends 15 mm proximal and 5 mm distal to the major duodenal papilla. The majority of labeled cells in a ganglion appeared within 10 mm around the major duodenal papilla as a cluster of 2-3 cells (Fig. 1), whereas the frequency of the labeled neurons decreased with the distance from
the papilla as a single cell. Labeled cells were occasionally found in the myenteric plexus up to about 15 mm proximal to the papilla. However, we were unable to find any labeled cells in the submucous plexus of this segment of the duodenum.

The frequency of FB-labeled neurons in the myenteric plexus of the mesenteric and anti-mesenteric portions of the proximal duodenum were 6.8% and 8.2% respectively, while those of the distal duodenum were 7.4% and 9.3%. However, there were no significant differences between the mesenteric and anti-mesenteric regions (Table 1, p > 0.1).

Double-labeling experiments by FB-injection and immunofluorescence revealed that the duodenal neurons retrogradely labeled from the gallbladder containing CGRP, GAL and VIP (Fig. 2). The immunoreactivity of the putative neuropeptides was observed in the nerve fibers, as well as in the nerve cell bodies of the ganglion. In spite of colchicine treatment, the peptide immunoreactivity within cell bodies was relatively lower than that observed in fibers. Although the actual frequency of the neuropeptidergic neurons projecting to the injection sites in the gallbladder from the myenteric plexus of the duodenum was not calculated, there was a colocalization of CGRP-immunoreactivity in approximately one tenth of the FB-labeled cells. Occasionally, neuropeptidergic cells without FB were also seen. The distribution and immunoreactivities of GAL and VIP were very similar to those of CGRP. FB-labeled neurons were randomly scattered in the ganglion without any special topographical localization in conjunction with neuropeptides. Control injections placed into the peritoneal cavity or on the gallbladder wall did not label myenteric neurons (data not shown).

DISCUSSION

After an intrinsic entero-biliary neural pathway was functionally shown (Wyatt, 1967, 1969), Mawe and Gershon (1989) provided the first morphological evidence for the existence of duodenal myenteric neurons projecting to the gallbladder in the guinea pig. Subsequently, Padbury et al. (1993) identified the projections of duodenal myenteric neurons to the sphincter of Oddi and of sphincter neurons to the gallbladder in the Australian possum. The present study demonstrates the presence of this intrinsic neural pathway from the duodenum to the gallbladder in the golden hamster. These duodenal neurons are mostly confined to the area around the major duodenal papilla (1.5 mm proximal and 5 mm distal to the papilla). This result shows that the pattern of confined localization of labeled neurons in the golden hamster is broadly similar to that in the guinea pig rather than that in the Australian possum, in which no labeled neurons were found in the first 9 mm of duodenum proximal to the sphincter (Mawe and Gershon, 1989; Padbury et al., 1993). Although little is known about the reason for the presence of the nerve supply from the duodenal myenteric plexus to the gallbladder, it is a reasonable postulation that the direct neural connection is needed for more precise and more effective synchrony between gastric peristalsis and the bile flow.

Our data on the frequency of nerve cell bodies of the myenteric plexus of the duodenum suggests that the mesenteric and anti-mesenteric portions of the duodenum equally contribute their neuronal projections to the gallbladder.

<table>
<thead>
<tr>
<th>Duodenal parts</th>
<th>FB-labeled cells</th>
<th>Total neurons</th>
<th>%</th>
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<tbody>
<tr>
<td>Proximal</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Mesenteric</td>
<td>2.8 ± 1.6</td>
<td>41.3 ± 19.3</td>
<td>6.8</td>
</tr>
<tr>
<td>Anti-mesenteric</td>
<td>3.0 ± 1.4</td>
<td>36.4 ± 10.4</td>
<td>8.2</td>
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<tr>
<td>Distal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesenteric</td>
<td>2.4 ± 0.9</td>
<td>33.1 ± 15.1</td>
<td>7.4</td>
</tr>
<tr>
<td>Anti-mesenteric</td>
<td>3.1 ± 1.2</td>
<td>33.5 ± 11.7</td>
<td>9.3</td>
</tr>
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Cell counts (Mean ± S.D.) were made in a 10 mm of the duodenal segment adjacent to the major duodenal papilla, 5 mm proximal and 5 mm distal to the papilla. Data from three animals.
Fig. 2. Micrographs of the double-labeled neurons with fast blue (A, D, G) and neuropeptides (B, E, H) in the same slices (A–C, D–F, G–I). The duodenal neurons retrogradely labeled from the gallbladder contained CGRP (B), GAL (E) and VIP (H). Neuropeptide immunoreactivity was observed in the nerve fibers (open arrow head) as well as the nerve cell bodies of the ganglion. Compared with the frequency of only FB-labeled cells (arrowheads), those of CGRP-, GAL- and VIP-containing neurons with FB (arrows) were quite low. Neuropeptidergic cells without FB (asterisks) were seen occasionally. C, F, and I are the cresyl violet stain of the first two fields in the CGRP, GAL and VIP immunoreactive sets. Scale bar = 50 μm.
Furthermore, we have provided the first anatomical evidence that some duodenal neurons containing CGRP, GAL and VIP project to the gallbladder. Although only a small population of labeled cells containing CGRP, GAL and VIP were observed, it should be noted that injected area was estimated to be no more than 20% of the gallbladder surface. The existence of CGRP, GAL and VIP immunoreactive fibers on the gallbladder from the myenteric plexuses of the duodenum suggests their involvement in the control of gallbladder motility, although this needs to be verified by means of physiological and pharmacological experimentation.

In summary, a direct neuronal connection from the duodenum to the gallbladder exists in the hamster, and these neurons exert their influence on the gallbladder via neuropeptides such as CGRP, GAL and VIP.

REFERENCES


