Catecholaminergic neurons in rat dorsal motor nucleus of vagus project selectively to gastric corpus

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GASTRIC RELAXATION refers to the behavior of the stomach during food ingestion and is characterized by a pronounced capacity to receive large increases in volume with only a slight increase in gastric pressure (1). The vagus nerve provides an essential role in the mediation of this relaxation reflex via an increase in nonadrenergic noncholinergic (NANC) inhibitory innervation to the stomach (1, 20, 29, 34). Anatomic and physiological evidence points to a possible role of the NO synthase (NOS)-IR positive DMV neurons in vagally mediated gastric relaxation. We (43) have shown recently that a subpopulation of DMV neurons containing NOS-IR projects selectively to the gastric fundus.

The dorsal motor nucleus of the vagus (DMV) supplies parasympathetic motor preganglionic fibers to the viscer and is involved in the central nervous system (CNS) control of gastric motility (10). Recently, we (29) have provided physiological evidence that at least two types of vagal motoneurons are involved in the gastric relaxation that follows esophageal distension.

Several studies (2, 3, 15, 17–19, 21, 22, 28, 35, 40, 43) have shown that the DMV contains neurons with discrete neurochemical phenotypes. These discrete neurochemical subpopulations could potentially provide differential CNS modulation of specific vago-vagal reflexes involved in, for example, gastric relaxation.

It is well established that the DMV in general and its caudal portion in particular are composed of neurons that show tyrosine hydroxylase immunoreactivity (TH-IR) (2, 13, 22, 28, 35, 37). The pattern of distribution of dopamine-β-hydroxylase (DBH) activity overlaps the distribution of TH-IR in the caudal dorsal brain stem (6, 13, 28). Recently, Willing and Berthoud (37) reported that the population of DBH and TH-IR neurons in the dorsal vagal complex (i.e., DMV and nucleus of the solitary tract, NTS) were almost identical in number and distribution, arguing that the TH-IR positive neurons in the DMV are capable of synthesizing norepinephrine. These TH-IR positive caudal DMV neurons have been reported (2, 22) to display choline acetyltransferase activity, making them likely candidates for vagal motoneurons that project to peripheral targets.

Studies (4, 19) have also focused on the presence and role of nitric oxide (NO) in the brain stem vagal nuclei. Anatomic and physiological evidence points to a possible role of the NO synthase (NOS)-IR positive DMV neurons in vagally mediated gastric relaxation. We (43) have shown recently that a subpopulation of DMV neurons containing NOS-IR projects selectively to the gastric fundus.

Immunocytochemical detection of the immediate-early genes encoding for the c-Fos protein allows detection of those neurons activated after a wide variety of stimuli. Increased c-Fos expression in response to administration of the gastric relaxant CCK or after gastric distension has been reported (27, 37) in neurons located in areas of the caudal dorsal brain stem that include the DMV and NTS.

The goals of this study were threefold. We aimed to (1) investigate whether the TH-IR positive DMV neu-

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rons explore whether TH- and NOS-IR positive neurons are colocalized within the same DMV neurons; and 3) examine whether TH-IR positive neurons are activated on esophageal distension.

**EXPERIMENTAL PROCEDURES**

A total of 47 Sprague-Dawley rats (25–35 days old) of either sex were used. Animals were subdivided into the following six groups. In group 1 (n = 6) or control, no injections were performed on the gastric walls. In group 2 (n = 8), tetramethylrhodamine isothiocyanate (TRITC)-filled latex microspheres were injected into the wall of the gastric fundus. In group 3 (n = 10), TRITC-filled latex microspheres were injected into the wall of the gastric corpus. In group 4 (n = 8), TRITC-filled latex microspheres were injected into the wall of the antral/pyloric area (see below). In group 5 (n = 10), double immunocytochemistry (TH and NOS) was carried out, and no injections were performed on the gastric walls. In group 6 (n = 5), esophageal distension followed by double immunocytochemistry (TH and c-Fos) took place, and no injections were performed on the gastric walls. Unless otherwise specified, all rats were injected with fluoresgor (Fluorochrome, Englewood, CO; 20 μg/1 ml saline/rat, ip) to label vagal preganglionic neurons innervating the subdiaphragmatic viscera allowing delineation of the boundaries of the DMV (24, 43).

Using a custom-made anesthetic chamber, rats were anesthetized deeply (abduction of foot pinch withdrawal reflex) with 2-bromo-2-chloro-1,1,1-trifluoroethane (halothane), and the abdominal and thoracic areas were shaved and cleaned with 70% ethanol. After an abdominal laparotomy, the stomach was freed from the liver and reflected gently to one side to facilitate access to the gastric wall. With a 5-μl Hamilton syringe, rats in groups 2, 3, and 4 were injected with TRITC-filled latex microspheres (1:4 vol/vol dilution with saline; 0.5–1 μl per injection, 5–10 sites per area) in the muscular and mucosal layers of the stomach (fundus, corpus, or antrum/pylorus, respectively). The syringe needle was inserted 2–3 mm into the stomach wall at an angle of 2–5° to facilitate the spread of beads within the stomach wall and to reduce the possibility of perforating the mucosa, perceived as a drop in the resistance opposing the needle penetration into the stomach wall. The laparotomy was then closed with 5-0 silk sutures, and the rats were allowed to recover for 5–15 days, permitting retrograde transport of the fluorescent marker as described previously (43).

Rats in group 6 were anesthetized with pentobarbital sodium (Nembutal; 75mg/kg, ip). The thoracic esophagus was then cannulated with a distension balloon inserted to the gastric wall covered by injection of rhodamine-coated beads was limited to ~5 mm². Therefore, this provided only a small, though representative, fraction of labeled DMV neurons projecting to the gastric areas of interest.

The portion of the DMV located caudal to obex was defined as the “caudal DMV” and the portion of DMV located rostral to the anterior tip of the area postrema as the “rostral DMV.” The area composed by the extension of the area postrema was defined as the “intermediate DMV.” Cell count values are given as means ± SE.

Cells were counted on alternate brain stem slices by two independent investigators who were unaware of the treatment. If the cell count differed by >10%, a third investigator then analyzed the brain stem slices. The final cell count was the mathematical average of the independent cell counts.

To minimize errors in the counting of DMV somata, we counted only those neurons in which the nucleus was clearly visible. Despite this precaution, we have to consider cell counts as best proportional estimates only, rather than absolute values, when comparing labeled subpopulations. This is of paramount importance because the surface of the stomach wall covered by injection of rhodamine-coated beads was limited to ~5 mm². Therefore, this provided only a small, though representative, fraction of labeled DMV neurons projecting to the gastric areas of interest.

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Control experiments were carried out to ensure that the antibody labeling was selective, namely: 1) incubation of primary or secondary antibodies only and 2) reaction of primary antibody with inappropriate secondary antibody. All
tests proved negative, indicating that the secondary antibodies were selective for their primary antibodies and that the antibodies themselves exhibited neither nonspecific binding nor excessive autofluorescence. Control experiments were also carried out to ensure that there was no leakage or fading of the fluorescence from the retrograde-labeled neurons due to the immunohistochemical staining process. This was accomplished by comparison of the number of retrograde-labeled neurons counted immediately after slicing and after the immunohistochemical staining process.

The experiments were carried out in accordance with the Henry Ford Health System guidelines for the care and use of laboratory animals. All efforts were made to minimize pain, reduce the number of animals used, and utilize alternatives to in vivo techniques, if available.

Photographs were taken using a SPOT digital camera mounted on a Nikon E400 fluorescent microscope. To reduce the fluorescent light exposure time and the consequent fading of the fluorescence labeling, slices were photographed in black and white using the appropriate filters, merged, and assigned compute-generated colors (SPOT software, Diagnostic Instruments, Sterling Heights, MI).

Materials. TRITC latex microspheres (rhodamine beads) were purchased from Lumafluor (Naples, FL). Nembutal was purchased from Abbot Laboratories (North Chicago, IL). All other chemicals were purchased from Sigma Chemical.

Composition of solutions. The fixative solution was composed of 32 g paraformaldehyde, 240 ml saturated picric acid, 5.25 g KH2PO4, 35.6 g Na2HPO4·7H2O, and 1,600 ml H2O. The composition of the PBS solution was as follows: 13.5 g NaCl, 40.2 g Na2HPO4·H2O, 2.04 g KH2PO4, and 1,500 ml H2O. The PBS-TX solution was composed of 2.25 ml Triton X-100 dissolved in 1,500 ml PBS buffer. The PBS-TX-BSA solution composition was 1 g BSA dissolved in 100 ml PBS-TX buffer.

Statistical analysis. Neuronal groups were compared using the χ2 test. The level of significance was set at P < 0.05.

RESULTS

Sections of brain stem containing the DMV were analyzed from ~2 mm caudal to the posterior tip of the area postrema to ~3 mm rostral to the anterior tip of the area postrema. The boundaries of the DMV were delineated clearly by the presence of fluorogold-IR neurons (19, 24, 43). Only those neurons with a distinct fluorogold-stained profile were counted. There were 998 ± 62, 2,283 ± 87, and 1,080 ± 59 fluorogold-positive neurons in the caudal, intermediate, and rostral DMV, respectively (n = 6 rats).
**Localization and projections of TH-IR neurons.** TH-IR neurons were distributed within the boundaries of the DMV (as determined by fluorogold-IR) as well as in the commissuralis, centralis, dorsalis, and medialis subnuclei of the NTS. Focusing on the TH-IR neurons in the DMV, the majority of the TH-IR neurons were located in the caudal DMV where they comprised 10.9 ± 0.6% of the total number of DMV neurons (i.e., 108 ± 12 TH-IR neurons of 998 ± 62 fluorogold-positive neurons; n = 6 rats). Although there were more fluorogold-labeled neurons in the intermediate portion of the DMV, there were fewer TH-IR positive DMV neurons present (i.e., 6 ± 1 TH-IR neurons of 2,283 ± 86 fluorogold-positive neurons; n = 6 rats). In the rostral portions of the DMV, the percentage of TH-IR DMV neurons was 4.4 ± 0.9% (i.e., 47 ± 7 TH-IR neurons of 1,080 ± 58 fluorogold-positive neurons; n = 6 rats). The total number of TH-IR neurons in the DMV would then constitute a value similar to that previously found (22), though somewhat lower than in a more recent study (39).

By applying retrograde-tracing techniques and immunohistochemical staining with specific antibodies against TH, we analyzed the rostro-caudal span of the DMV for colocalization of rhodamine-labeled and TH-IR positive neurons, i.e., TH-IR neurons projecting to the gastric regions of interest.

After injection of rhodamine in the corpus, 43 of the 368 rhodamine-labeled neurons in the caudal portion of the DMV colocalized with TH-IR (i.e., 13.7 ± 1.1%; n = 10 rats; P < 0.05 vs. fundus or antrum/pylorus). Conversely, after injection of rhodamine beads in the antrum/pylorus, 1 of the 41 rhodamine-labeled neurons in the caudal DMV also contained TH-IR (n = 8 rats); similarly after injection of rhodamine beads in the fundus, 9 of the 216 rhodamine-labeled neurons colocalized with TH-IR (n = 8 rats).

Figure 1 shows the distribution of dye-labeled neurons after injection of tracer in the gastric corpus, intraperitoneal injection of fluorogold, and immunohistochemical detection of TH. As can be noted in the micrograph (Fig. 1), most of the DMV neurons showing colocalization of dye and TH-IR were located in the caudal pole of the DMV whereas the majority of dye-labeled DMV neurons were in the intermediate portion of the DMV. In Fig. 1, A and B (showing caudal and intermediate brain stem section, respectively), DMV neurons show colocalization of rhodamine beads and TH-IR. In the rostral portion of the DMV (Fig. 1C), TH-IR positive neurons are located outside the fluorogold-defined boundaries of the DMV.

**Localization of TH- and NOS-positive neurons.** In the 10 brain stems analyzed, we never found any DMV neurons that contained both NOS- and TH-IR (Fig. 2), even in the caudal DMV, where both TH- and NOS-IR positive neurons are densely located. In fact, despite the observation that TH-IR and NOS-IR positive DMV neurons...
neurons were contained within the same region of the caudal DMV, they were never colocalized.

Figure 2 is a micrograph showing the distribution of TH- and NOS-IR positive neurons in the caudal portion of the DMV. As can be noted, within the fluorogold-delimited boundaries of the DMV, the TH-IR positive neurons are intermingled but not colocalized with the NOS-IR positive neurons. The separation of TH- and NOS-IR positive neurons is also present in the NTS area adjacent to the DMV where the NOS-IR positive neurons are located in patches dorsal to the TH-IR positive neurons.

Esophageal distension activates c-Fos expression. After esophageal distension for a period between 60 and 90 min (1 s on and 4 s off) we observed a consistent pattern of c-Fos expression in the caudal dorsal brainstem. At the level of the caudal DMV in all five brain stems analyzed, although c-Fos-positive neurons were present, we never found a DMV neuron that contained both c-Fos- and TH-IR (Fig. 3).

DISCUSSION

The present study provides evidence that 1) TH-IR positive neurons in the caudal DMV project selectively to the gastric corpus; 2) the TH-IR positive DMV neurons comprise a neuronal subpopulation distinct from the NOS-IR positive DMV neurons; and 3) TH-IR positive neurons in the DMV are not activated on esophageal distension.

Such evidence leads us to the following two conclusions. 1) The TH-IR positive neurons in caudal DMV constitute a subpopulation of preganglionic neurons distinct from the NOS-IR neurons. These TH-IR neurons do not seem to be implicated in the activation of NANC inhibitory pathways of the gastric receptive relaxation reflex activated by esophageal distension (29, 43). 2) The discrete population of TH-IR positive neurons in caudal DMV projecting to the corpus may then comprise the preganglionic motoneurons involved in gastric relaxation obtained via withdrawal of cholinergic tone (29, 34) or could constitute a subpopulation of dopaminergic neurons involved in the attenuation of stress or chemically induced ulcers (see Ref. 11 for review).

The percentage of TH-IR positive neurons in the caudal DMV reported in this study, i.e., 11% of the fluorogold-labeled neurons, is similar to that found previously by Manier et al. (22), though slightly lower than in other reports (35, 39), supporting the validity of our labeling techniques and cell-counting methods.

Fig. 3. Coronal view of the caudal brain stem showing the distribution within the DMV (outlined by the fluorogold positive labeling) of TH- and c-Fos-IR positive neurons. Computer generated colors indicate the following: blue, fluorogold; green, TH-IR; red, c-Fos-IR; light blue, colocalization of fluorogold and TH-IR. Note that c-Fos positive neurons (thin arrows) are either outside the DMV or inside the DMV but do not contain TH (thin convex arrows). In fact, TH-IR neurons in the DMV do not colocalize with c-Fos (thick arrows).

In this study, we report that ~14% of vagal neurons projecting to the rat gastric corpus contain TH-IR. The importance of such a relatively low percentage of
TH-IR neurons in the DMV should not be underestimated, because single vagal motoneurons project extensively to adjacent areas of the gastrointestinal tract (14) and could thus influence selectively similar functions.

Our data showing lack of colocalization of TH- and NOS-IR in caudal DMV neurons extend a previous observation by Ohta and colleagues (23). In that study (23), evidence was provided that NOS neurons did not colocalize with catecholaminergic neurons in the adjacent medial NTS subnucleus.

Using both in vitro and in vivo techniques, several groups have shown recently that a vast array of electrical (36, 38), physiological (27, 29), or mechanical (8, 25, 37) stimuli can activate DMV motoneurons and induce gastric relaxation (8, 27, 29). Some of these stimuli, i.e., peripheral injection of CCK (27) or gastric (37) or esophageal distension (29), selectively activate the caudal DMV neuronal population.

Vagally mediated cholinergic stimuli increase gastric functional parameters, including tone and motility (10). In several studies (9, 30, 31, 41, 42), it has been demonstrated that activation of presynaptic α2-adrenoceptors decreases vagally evoked ACh release to the stomach and that exogenous application of norepinephrine decreases ACh release from vagal terminals in both the corpul (30) and antral portions of the stomach (32). In addition, the decrease in gastric motility that follows peripheral CCK administration involves the activation of a vagal-dependent mechanism (12, 26, 27). It is established that both endogenous and exogenous CCK stimulate distension-sensitive vagal afferents, producing distension-like effects on vagal neurons (26) and expression of c-FOS in TH-IR positive DMV neurons (27).

Using an esophageal balloon distension protocol proven to induce gastric relaxation (29), we were unable to activate c-Fos expression in TH-IR positive caudal DMV neurons. Our data thus suggest that the origin of the adrenergic modulation of ACh release from vagal terminals must be found in areas other than the caudal DMV.

Because our esophageal stimulation paradigm did not activate TH-IR caudal DMV neurons (otherwise we would have observed an increase in c-Fos activity), the following two scenarios may be considered.

First, TH-IR positive neurons in the caudal DMV may be implicated in withdrawal of cholinergic tone by an activation of GABA terminals via a mechanism unrelated to esophageal distension. In fact, adrenergic activation of GABA terminals via a mechanism may be implicated in withdrawal of cholinergic tone by the following two scenarios may be considered.

In conclusion, we have shown that a significant percentage of caudal DMV neurons projecting to the stomach corpus contain TH-IR and are not colocalized with NOS-IR positive neurons. In addition, we suggest that the TH-IR positive neurons may be involved in the withdrawal of cholinergic tone to the gastric corpus.

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