Intracisternal TRH analog induces Fos expression in gastric myenteric neurons and glia in conscious rats

MARCEL MIAMPAMBA,1 HONG YANG,1 KEITH A. SHARKEY,2 AND YVETTE TACHÉ1
1CURE: Digestive Diseases Research Center, Veterans Affairs Greater Los Angeles Healthcare System, and Department of Medicine and Brain Research Institute, University of California, Los Angeles, California 90073; and 2Neuroscience Research Group, Department of Physiology and Biophysics, University of Calgary, Calgary, Alberta, Canada T2N 4N1

Received 25 July 2000; accepted in final form 13 December 2000

Intracisternal TRH analog induces Fos expression in gastric myenteric neurons and glia in conscious rats. Am J Physiol Gastrointest Liver Physiol 280: G979–G991, 2001.—Activation of gastric myenteric cells by intracisternal injection of the stable thyrotropin-releasing hormone (TRH) analog RX-77368, at a dose inducing near maximal vagal cholinergic stimulation of gastric functions, was investigated in conscious rats. Fos immunoreactivity was assessed in gastric longitudinal muscle-myenteric plexus whole mount preparations 90 min after intracisternal injection. Fos-immunoreactive cells were rare in controls (~1 cell/ganglion), whereas intracisternal RX-77368 (50 ng) increased the number to 24.8 ± 1.8 and 26.8 ± 2.2 cells/ganglion in the corpus and antrum, respectively. Hexamethonium (20 mg/kg sc) prevented Fos expression by 90%, whereas atropine (2 mg/kg sc) had no effect. The neuronal marker protein gene product 9.5 and the glial markers S-100 and glial fibrillary acidic proteins showed that RX-77368 induced Fos in both myenteric neurons and glia. Vesicular ACh transporter and calretinin were detected around the activated myenteric neurons. These results indicated that central vagal efferent stimulation by intracisternal RX-77368 activates gastric myenteric neurons as well as glial cells mainly through nicotinic ACh receptors in conscious rats.

RX-77368; immediate-early gene; atropine; hexamethonium; vesicular acetylcholine transporter; enteric nervous system

MEDULLARY THYROTROPIN-RELEASING hormone (TRH) plays a physiological role in the vagal regulation of gastric function (22, 27, 45). TRH-containing fibers densely innervate the dorsal vagal complex including the nucleus of the solitary tract and dorsal motor nucleus of the vagus (DMN) where nerve terminals synapson dendrites of motoneurons projecting to the stomach (25, 36). This innervation originates exclusively from TRH-immunoreactive cell bodies located in the raphe pallidus, raphe obscurus, and parapyramidal region of the ventral medulla (25). In vitro electrophysiological studies showed that TRH activates neurons in the DMN through direct postsynaptic action (47) and that intracisternal injection of TRH or the stable TRH analog RX-77368 increases gastric vagal efferent discharges (32). Functional studies also established that intracisternal TRH or acute exposure to cold results in vagal cholinergic stimulation of gastric emptying through activation of TRH receptor 1 (26, 27, 50) expressed on DMN neurons (12). It is well known that the myenteric plexus embedded in the stomach wall plays an essential role in gastric emptying (13) and receives extrinsic regulatory inputs (39, 52, 53). Electrophysiological studies (39) have shown that electrical stimulation of the gastric vagus induces widespread activation of gastric myenteric neurons through nicotinic pathways in the guinea pig isolated stomach as monitored by electrophysiological recording. Similarly, electrical stimulation of the cervical vagus results in neuronal activation within the gastric myenteric plexus in anesthetized rats as shown by Fos expression (52, 53). However, data mentioned above result mainly from studies (52, 53) using electrical stimulation of the vagus nerve at high frequencies where results mainly from studies (52, 53) using electrical stimulation of the vagus nerve at high frequencies in anesthetized animals. So far, the influence of central vagal efferent activation on the activity of gastric myenteric neurons in conscious rats is still unknown.

The present study was undertaken to assess the activity of gastric myenteric neurons in response to intracisternal injection of RX-77368 in conscious rats. RX-77368 was used at the doses previously established (18, 26, 27, 45) to induce near maximal vagal-dependent, atropine-sensitive stimulation of gastric motor function, acid secretion, and blood flow in rats. Immunohistochemical detection of the nuclear protein Fos in the gastric myenteric ganglia was used to monitor the synaptic activation of myenteric cells (23). The involvement of cholinergic pathways in the mechanisms leading to myenteric cell activation, after intracisternal injection of RX-77368, was also investigated by immunohistochemistry using an anti-vesicular ACh transporter (VAChT) antibody. VAChT is considered to be a suitable marker of cholinergic innervation in the central as well as peripheral nervous system (3, 38). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests and other correspondence: M. Miampamba, CURE: DDRC, Veterans Affairs Greater Los Angeles Healthcare System, Bldg. 115, Rm. 203, 11301 Wilshire Blvd., CA 90073 (E-mail: mmiamampam@ucla.edu).

The American Physiological Society
role of nicotinic and muscarinic receptors in central RX-77368-induced changes in activity in the gastric myenteric plexus was investigated using hexamethonium, a selective ganglionic blocking agent of peripheral nicotinic transmission, or atropine, a muscarinic receptor antagonist. To delineate the neuronal identity of activated myenteric cells, we performed immunohistochemical double labeling of Fos with protein gene product 9.5 (PGP 9.5), a marker for neuronal cell bodies and axons in the central and peripheral nervous system (20). The immunohistochemical colocalization of Fos protein with calretinin, a calcium-binding protein synthesized in neurons (1), was also assessed in the gastric myenteric plexus. The possible presence of Fos immunoreactivity in nonneuronal supportive cells within the gastric myenteric ganglia was examined using a combination of Fos with either S-100 protein, which is present in glial as well as other supportive cells, or glial fibrillary acidic protein (GFAP), a specific marker of glial cells (21, 30).

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Harlan, San Diego, CA) weighing 260–300 g were housed in groups of four animals per cage and kept under controlled conditions of 12:12-h light-dark cycle with room temperature maintained at 22 ± 1°C. The rats had free access to Purina Laboratory Chow (Ralston Purina, St. Louis, MO) and tap water for at least 1 wk before the experiments. Food and sawdust bedding, but not water, were withdrawn 24 h before the treatments, which were carried out between 9 and 12 AM. Fifty-four rats were used in the study. The procedures used were approved by the Veterans Affairs Animal Component of the Research Protocol number 98-080-08.

Drugs and Treatments

Drugs. RX-77368 [pGlu-His-(3,3′-dimethyl)-Pro-NH₂]; Ferring Pharmaceuticals, Feltham, Middlesex, UK] was dissolved in 0.1% BSA and sterile saline (0.9% NaCl) at the concentration of 300 ng/10 μL, separated in aliquots, and kept frozen at −80°C. Hexamethonium chloride and atropine sulfate salt (both from Sigma Chemical, St. Louis, MO) were diluted in saline as well as the stock solution of RX-77368 a few minutes before administration.

Antibodies and reagents. The following primary antibodies were used: polyclonal rabbit anti-Fos (Fos Ab-5; Oncogene Research Products, Cambridge, MA); polyclonal rabbit and monoclonal mouse anti-human PGP 9.5 (Ultraclone, Isle of Wight, UK); polyclonal rabbit anti-VACH (Phoenix Pharmaceuticals, Mountain View, CA); polyclonal rabbit anti-calretinin (Swant, Swiss Antibodies, Bellinzona, Switzerland); monoclonal mouse anti-Fos (TF161, Dr. K. Riabowol, University of Calgary, Calgary, Alberta, Canada); monoclonal mouse anti-GFAP (Chemicon International, Temecula, CA); and monoclonal mouse anti-S-100 protein (Biomedia, Foster City, CA). The secondary antibodies used were as follows: biotinylated goat anti-rabbit IgG, donkey anti-mouse IgG conjugated to FITC or tetramethylrhodamine isothiocyanate (TRITC), and goat anti-rabbit IgG conjugated to TRITC (Jackson ImmunoResearch, West Grove, PA); goat anti-rabbit IgG conjugated to FITC (Insecta, Stillwater, MN); and sheep anti-mouse IgG conjugated to fluorescent cyanine dye Cy3 and normal goat serum (both from Sigma Chemical). Nifedipine, Triton X-100, and hydrogen peroxide were purchased from Sigma Chemical. The Triton X-100 EliA avidin biotin complex kit, which contains avidin and biotinylated horseradish peroxidase reagents, and the diaminobenzidine peroxidase substrate kit for immunoperoxidase staining were purchased from Vector Laboratories (Burlingame, CA).

Intracisternal injection. The intracisternal injection was performed acutely as previously described (27). Under short (2–3 min) enflurane anesthesia (5% vapor concentration in oxygen; Ethrane, Ohmeda Pharmaceutical, Liberty Corner, NJ), the head of the rat was immobilized with ear bars of a Kopf stereotaxic frame (model 900) and the atlantooccipital membrane was punctured with a 50-μl Hamilton microsyringe. The successful insertion of the needle into the cisterna magna was verified by the aspiration of a clear cerebrospinal fluid into the syringe. Ten microliters of sterile saline or RX-77368 solution were injected, and then the animal was returned to its individual cage where recovery from anesthesia occurred within 2–4 min.

Experimental Protocols

Effects of RX-77368 on Fos expression in gastric myenteric plexus. Rats were injected intracisternally with either saline (n = 6) or RX-77368 at a dose of 30 (n = 4) or 50 ng (n = 7) and euthanized 90 min later. Two other groups received either intracisternal saline (n = 3) or RX-77368 (50 ng; n = 4) and were euthanized 60 min later. Gastric tissues were immediately removed and processed for immunohistochemistry. The time periods (60 and 90 min) were selected based on a previous study from Zheng et al. (53) showing that Fos immunoreactivity was present in almost all gastric myenteric neurons 60 min after a 30-min period of electrical vagal stimulation in anesthetized rats.

Effects of nicotinic and muscarinic receptor blockade. Rats were injected subcutaneously (0.26–0.30 ml) with either saline (n = 4) or hexamethonium at a dose of 10 (n = 8) or 20 mg/kg (n = 4), and 30 min later RX-77368 (50 ng) was injected intracisternally. In other groups, rats received no pretreatment (n = 2) or were injected subcutaneously (0.26–0.30 ml) with atropine at a dose of 1 (n = 3) or 2 mg/kg (n = 5), and 30 min later RX-77368 (50 ng) was injected intracisternally. Atropine was also injected subcutaneously (2 mg/kg), followed 30 min later by an intracisternal injection of saline (10 ml) (n = 4). Rats were killed 90 min after the intracisternal injection. Gastric tissues were immediately collected and processed for immunohistochemistry.

Tissue Preparation

Animals were killed by decapitation, and the stomach was immediately removed, placed in PBS (pH 7.4) containing nifedipine (10⁻⁶ M), and opened along the smaller curvature. The stomach was then pinned flat in a Sylgard-coated petri dish (Sylgard 184, Dow Corning, Midland, MI) and fixed overnight in Zamboni’s fixative (pH 7.4) at 4°C. After fixation, the gastric tissue was rinsed (3 times for 10 min each) in PBS, and the corpus was separated from the antrum based on our (9, 34) previous findings that intracisternal TRH or RX-77368...
increased high-amplitude contractions in these regions through vagal pathways.

**Immunohistochemistry**

*Fos immunostaining.* Immunohistochemical labeling was performed using the avidin-biotin peroxidase immunostaining procedure as previously described (28). Briefly, LMMP whole mount preparations from the corpus or antrum were rinsed (3 times for 10 min each) in PBS and incubated for 30 min at room temperature with PBS containing 0.3% hydrogen peroxide to remove endogenous peroxidase activity. After a final rinse (3 times for 5 min each) in PBS, tissues were incubated for 1 h at room temperature with normal goat serum (3%) in PBS containing 0.3% Triton X-100 as a blocking solution to prevent the nonspecific binding before being placed in primary antibody. Tissues were then incubated for 48 h at 4°C with Fos Ab-5 (1:10,000) diluted in blocking solution. After the primary antibody, tissues were rinsed (3 times for 10 min each) in PBS and incubated for 2 h at room temperature with biotinylated goat anti-rabbit secondary antibody (1:500; Jackson ImmunoResearch) diluted in PBS containing 0.3% Triton X-100. Finally, tissues were rinsed (3 times for 10 min each) in PBS and processed using the standard biotin-avidin-horseradish peroxidase methodology (15). Immunohistochemical controls routinely performed involved incubation of tissues in blocking solution followed by antibody diluent in place of primary antibody and processed as described above. Tissues were examined using a light microscope (Axioskop, Carl Zeiss, Thornwood, NY) and photographed (Kodak technical pan film). No positive staining was observed in controls.

**Double Labeling and Microscopy**

The general procedure involved Fos staining with either PGP 9.5, VACHT, calretinin, GFAP, or S-100 protein in LMMP whole mount preparations from corpus or antrum examined using an indirect immunofluorescence technique as previously described (28, 29). For the double-labeling protocol, primary as well as secondary antibodies were mixed before use. The staining provided by each primary antibody (monoclonal or polyclonal antibody) was visualized using a specific secondary antibody conjugated to either FITC or Cy3/rhodamine (TRITC). For the Fos and VACHT or Fos and calretinin double-labeling studies, Fos-immunoreactive cells in the myenteric ganglia were viewed using fluorescence optics with a TRITC filter, whereas VACHT or calretinin immunoreactivity was viewed using fluorescence optics with an FITC filter. To analyze the double-labeling Fos and GFAP or Fos and S-100 protein, Fos-immunoreactive cells in the myenteric ganglia were viewed using fluorescence optics with an FITC filter, whereas GFAP or S-100 protein immunoreactivity was viewed using fluorescence optics with a TRITC filter. Double-labeling Fos and PGP 9.5 analysis was performed using the same procedure mentioned above. When a mouse monoclonal anti-Fos (TF161) was combined with a rabbit polyclonal anti-PGP 9.5, Fos-immunoreactive cells in the myenteric ganglia were viewed using fluorescence optics with a TRITC filter, whereas PGP 9.5 immunoreactivity was viewed using fluorescence optics with a FITC filter. Conversely, Fos-immunoreactive cells in the myenteric ganglia were viewed using fluorescence optics with a FITC filter, whereas PGP 9.5 immunoreactivity was viewed using fluorescence optics with a TRITC filter. In all studies, fluorescence optics with both filters (TRITC/FITC) allowed dual-labeling analysis by overlapping both labelings obtained in the same gastric LMMP whole mount preparation. Fluorescence was viewed and images captured on a Zeiss LSM 510 laser scanning microscope. For each examined gastric LMMP whole mount preparation, three different stains detected by Cy3 and TRITC, FITC, or TRITC and FITC filters were viewed and captured using the computerized image analysis system coupled to the Zeiss LSM laser scanning microscope. The procedure allowed an accurate analysis of single vs. double immunohistochemical labeling on the same tissue. Data are presented as the combination of three images for each double immunohistochemical staining.

*Fos with PGP 9.5, VACHT, or calretinin staining.* Gastric LMMP whole mount preparations dissected from the corpus or antrum were rinsed (3 times for 10 min each) with PBS containing 0.1% Triton X-100 (PBS-T) and incubated in the blocking solution as described above. Tissues were then incubated for 48 h at 4°C with mouse monoclonal Fos antibody (TF161, 1:500) alone or combined with the following rabbit polyclonal antibodies: anti-human PGP 9.5 (1:500), anti-VACHT (1:500), or anti-calretinin (1:500). Because the immunohistochemical detection of Fos protein was performed using main the polyclonal Fos antibody (Fos Ab-5), LMMP whole mount preparations from both corpus and antrum were also incubated separately with Fos Ab-5 antibody alone or combined with the mouse monoclonal anti-human PGP 9.5. Tissues were then washed (3 times for 10 min each) with PBS-T and incubated for 1 h at room temperature with sheep anti-mouse IgG conjugated to Cy3 (1:100) or donkey anti-mouse IgG conjugated to FITC (1:100) alone or combined with goat anti-rabbit IgG conjugated to FITC (1:50) or goat anti-rabbit IgG conjugated to TRITC (1:100). All antibodies were diluted in PBS-T containing 0.1% BSA (antibody diluent). Tissues were given a final wash (3 times for 10 min each) with PBS-T and mounted in bicinephosphate-buffered glycerol (pH 8.6). Although the specificity of the primary antibodies Fos Ab-5, TF161, PGP 9.5, VACHT, and calretinin has been previously demonstrated (33, 38, 41, 42), controls were performed by incubating a few LMMP whole mount preparations in antibody diluent in place of the primary antibodies and processed as described above. Tissues were examined and photographed using a laser scanning microscope. No positive staining was seen in these controls.

*Fos with GFAP or S-100 protein staining.* LMMP whole mount preparations from corpus or antrum were rinsed (3 times for 10 min each) with PBS-T and incubated for 1 h at room temperature with a blocking solution before being incubated for 48 h at 4°C with Fos Ab-5 (1:5,000) alone or combined with mouse anti-GFAP (1:1,000) or mouse anti-S-100 protein (1:500). Tissues were then washed (3 times for 10 min each) with PBS-T and incubated for 1 h at room temperature with goat anti-rabbit IgG conjugated to FITC or goat anti-rabbit IgG conjugated to TRITC alone or combined with sheep anti-mouse IgG conjugated to Cy3 or donkey anti-mouse IgG conjugated to FITC. Antibodies were diluted in antibody diluent as above. After a final wash (3 times for 10 min each) with PBS-T, tissues were mounted in bicinephosphate-buffered glycerol (pH 8.6). Controls were performed by incubating a few LMMP whole mount preparations in antibody diluent instead of the primary antibodies and processed as described above. Finally, tissues were examined and photographed using a laser scanning microscope. No positive staining was seen in these controls.

**Quantitative Analysis and Statistics**

To avoid a possible discrepancy in the number of Fos-immunoreactive nuclei revealed by the monoclonal and polyclonal Fos antibodies, we blindly quantified the number of...
Fos-immunoreactive nuclei per myenteric ganglion in gastric tissues stained only with Fos Ab-5 using the avidin-biotin peroxidase immunostaining procedure or indirect immunofluorescence staining. An exception was made for the number of Fos-immunoreactive nuclei counted within the VACHT-immunoreactive cell bodies. Indeed, no mouse monoclonal antibody raised against VACHT was available. Mouse monoclonal Fos antibody (TF161) was then combined with rabbit polyclonal antibody raised against VACHT to study the colocalization of both markers. In each animal, the number of Fos-immunoreactive nuclei as well as VACHT-immunoreactive cell bodies expressing Fos-positive cells was counted in 25 myenteric ganglia randomly selected in the corpus or antrum and expressed as a mean number per myenteric ganglion. Myenteric ganglia were identified as clearly delineated groups of neurons separated by well-defined internodal fiber tracts in which neurons were absent (14, 28). The mean number of Fos-immunoreactive nuclei or VACHT-immunoreactive cell bodies expressing Fos per myenteric ganglion from each animal was used to generate a mean number for each experimental group. Data are expressed as means ± SE of the number of Fos-immunoreactive nuclei or VACHT-immunoreactive cell bodies expressing Fos per myenteric ganglion and were analyzed using one-way ANOVA. Differences between mean values were assessed by a Student-Newman-Keuls multiple-comparison test, and P < 0.05 was considered significant.

RESULTS

Intracisternal Injection of RX-77368 Induced Fos Expression in Gastric Myenteric Ganglia

In rats injected intracisternally with saline, gastric LMMP whole mount preparations incubated with polyclonal rabbit Fos antibody showed no or few ganglionic Fos-immunoreactive cells in the corpus (Fig. 1A) or antrum (Fig. 1B) at 90 min. RX-77368 (30 or 50 ng ic) induced widespread Fos expression in the myenteric ganglia within the corpus (Fig. 1, C and E) and antrum (Fig. 1, D and F) examined 90 min later in conscious rats. RX-77368 (30 or 50 ng) injected intracisternally induced a similar increase in the number of Fos-immunoreactive nuclei in the myenteric ganglia of both the corpus and antrum (Table 1). No significant differences in the number of Fos-immunoreactive nuclei per myenteric ganglion were observed within the corpus and antrum by comparing the effects of RX-77368 at both doses (Table 1). A similar level of Fos expression was also observed at 60 min after intracisternal injection of RX-77368 (50 ng) (Fig. 1, E and F; Table 1). All further data were obtained from tissues examined 90 min after the intracisternal injection of RX-77368 at 50 ng.

Effect of Hexamethonium and Atropine on Fos Expression in Gastric Myenteric Ganglia Induced by Intracisternal RX-77368 Injection

The number of Fos-positive cells increased in the myenteric ganglia after intracisternal injection of RX-77368 in rats pretreated with saline (Fig. 2). The morphology and shape of the observed Fos-immunoreactive nuclei were similar to that found above (Fig. 1, E and F). Fos expression induced by intracisternal RX-77368 was dose dependently inhibited by pretreatment with hexamethonium (10 or 20 mg/kg sc) as shown by the low number of Fos-immunoreactive cells per myenteric ganglion in the corpus (7.6 ± 0.6 and 2.8 ± 0.9, respectively) and antrum (9 ± 0.5 and 3.6 ± 0.3, respectively) compared with saline-pretreated rats injected intracisternally with RX-77368 (26.3 ± 2 and 26.7 ± 1.9 Fos-immunoreactive cells myenteric/ganglion for corpus and antrum, respectively) (Fig. 2). Hexamethonium at 20 mg/kg inhibited Fos expression in the corpus and antrum by 89.3% and 86.5%, respectively, and was more potent (P < 0.02) than at 10 mg/kg (Fig. 2). Hexamethonium (10 or 20 mg/kg sc) alone did not induce Fos expression in gastric myenteric ganglia (not shown).

Intracisternal injection of RX-77368 (50 ng) in rats triggered Fos expression in both corpus and antrum examined 90 min later (Fig. 3, A and B, Table 2).
Table 1. Intracisternal injection of TRH analog RX-77368 induced Fos expression in gastric myenteric cells

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Corpus (60 min)</th>
<th>Antrum (60 min)</th>
<th>Corpus (90 min)</th>
<th>Antrum (90 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0 ± 0(3)</td>
<td>0.1 ± 0.1(3)</td>
<td>0.5(6)</td>
<td>1.1 ± 0.4(5)</td>
</tr>
<tr>
<td>RX-77368 (30 ng)</td>
<td>20.5 ± 3.7*(4)</td>
<td>24.8 ± 1.8*(7)</td>
<td>26.5 ± 1.6*(4)</td>
<td>26.8 ± 2.2*(7)</td>
</tr>
<tr>
<td>RX-77368 (50 ng)</td>
<td>25.9 ± 0.8*(4)</td>
<td>24.8 ± 1.8*(7)</td>
<td>26.5 ± 1.6*(4)</td>
<td>26.8 ± 2.2*(7)</td>
</tr>
</tbody>
</table>

Values are means ± SE for the no. of Fos-immunoreactive nuclei/myenteric ganglion quantified as outlined in Quantitative Analysis and Statistics (in MATERIALS AND METHODS); the no. of rats is given in parentheses. Saline or RX-77368 was injected intracisternally under short anesthesia, and conscious rats were then euthanized 60 or 90 min later. The gastric corpus and antrum were processed for Fos immunohistochemistry using the polyclonal rabbit antibody raised against Fos (Fos Ab-5). The avidin-biotin peroxidase immunostaining procedure was used for Fos detection. TRH, thyrotropin-releasing hormone. *P < 0.001 compared with intracisternal saline-injected rats (ANOVA followed by a Student-Newman-Keuls multiple-comparison test).

Atropine pretreatment (1 or 2 mg/kg sc) did not significantly affect the number Fos-immunoreactive cells per myenteric ganglion in both corpus (Fig. 3, C and E) and antrum (Fig. 3, D and F), respectively (Table 2).

Neuronal Identity of Gastric Myenteric Fos-Immunoreactive Cells: Double Labeling Fos and PGP 9.5

Fos-immunoreactive nuclei were rare or absent in the gastric LMMP whole mount preparations from rats injected intracranially with saline using either Fos Ab-5 or monoclonal mouse anti-Fos (TF161) alone or combined with the neuronal marker PGP 9.5 (not shown). Double labeling TF161 and PGP 9.5 in gastric tissues collected after RX-77368 injection revealed that 100% of ganglionic Fos-immunoreactive nuclei were localized within the nerve cell bodies labeled with PGP 9.5 as depicted in the corpus (Fig. 4E). In contrast, double labeling Fos Ab-5 and PGP 9.5 revealed the presence of Fos-immunoreactive nuclei in neurons as well as in a small proportion of nonneuronal cells within the myenteric ganglia (Fig. 4F). Immunostaining with PGP 9.5 showed axons and nerve fibers as well as nerve cell bodies in myenteric ganglia (Fig. 4, C and D) in which Fos immunoreactivity was colocalized (Fig. 4, E and F).

Double Labeling Fos and VACHT

The cholinergic immunoreactivity in ganglionic Fos-immunoreactive neurons was assessed using the monoclonal mouse Fos antibody (TF161) combined with the polyclonal rabbit VACHT antibody. In rats injected intracisternally with saline, Fos-immunoreactive nuclei were absent in the gastric myenteric ganglia, whereas abundant nerve and varicose fibers resembling nerve terminals immunoreactive to VACHT were visible within the ganglia in the corpus and antrum (not shown). In contrast, RX-77368 injected intracisternally induced Fos expression in the ganglionic myenteric neurons as shown in the corpus (Fig. 5A) and antrum (Fig. 5B). VACHT immunostaining revealed abundant varicose fibers and terminals as well as a few cell bodies immunoreactive to VACHT in the myenteric ganglia within the corpus (Fig. 5C) and antrum (Fig. 5D). Double labeling Fos and VACHT showed that varicose fibers resembling nerve terminals and/or intrinsic cholinergic nerve fibers immunoreactive to VACHT were visible around all Fos-positive cells in both the corpus (Fig. 5E) and antrum (Fig. 5F). All ganglionic VACHT-immunoreactive cell bodies were also Fos positive (Fig. 5, E and F) with an approximately similar number per ganglion in the corpus (3.9 ± 0.6) and antrum (3.4 ± 0.3).

Double Labeling Fos and Calretinin

The double labeling was performed using a combination of a monoclonal mouse anti-Fos (TF161) with a polyclonal rabbit anti-calretinin. A dense network of nerve fibers and varicosities immunoreactive to calretinin was labeled in the corpus and antrum from control rats (not shown). In addition, Fos-immunoreactive nuclei were absent in these tissues, whereas approximately one to two calretinin-immunoreactive cell bodies were randomly counted in each examined myenteric ganglion in controls (not shown). RX-77368 injected intracisternally induced widespread Fos ex-
expression in the myenteric ganglia within the corpus (Fig. 6A) and antrum (Fig. 6B). Calretinin-immunoreactive nerve fibers and varicosities as well as nerve cell bodies were also present in the myenteric ganglia within the corpus (Fig. 6C) and antrum (Fig. 6D). Double labeling of Fos and calretinin showed a dense network of nerve fibers and varicosities immunoreactive to calretinin encircling 100% of Fos-immunoreactive neurons in the corpus (Fig. 6E) and antrum (Fig. 6F). Occasionally, the small proportion of myenteric nerve cell bodies immunoreactive to calretinin was also immunoreactive to Fos (Fig. 6F). Of ~18 Fos-immunoreactive neurons labeled with the monoclonal anti-Fos (TF161) and located at random in gastric myenteric ganglia after intracisternal RX-77368, one to two nerve cell bodies were also calretinin positive (Fig. 6F).

Glial Identity of Gastric Myenteric Fos-Immunoreactive Cells: Double Labeling Fos and GFAP

The staining was performed with Fos Ab-5 and monoclonal mouse anti-GFAP antibodies in the gastric LMMP whole mount preparations from saline (not shown) as well as RX-77368-injected rats (Fig. 7). GFAP immunoreactivity formed a plexus like-network that surrounded nonimmunoreactive cells, presumably neurons (Fig. 7, C and D). Immunostained GFAP-containing cells had extremely elongated cytoplasmic extensions, and the perikaryal cytoplasm was too scanty to properly discern the perikaryon (Fig. 7, C and D). Fos expression induced by RX-77368 (Fig. 7, A and B) was present in the glial cell network (Fig. 7, C and D) in close association with activated neurons in the corpus (Fig. 7E) and antrum (Fig. 7F).

Double Labeling Fos and S-100 Protein

Fos Ab-5 and monoclonal mouse S-100 protein antibodies were used. In saline (not shown) as well as RX-77368-injected rats (Fig. 8), S-100 protein-positive cell clusters were clearly visible as a bulbous group of immunolabeled strands around nonimmunoreactive perikarya (Fig. 8, C and D). Whereas the staining of GFAP displayed a network of thin bundles (Fig. 7, C and D), that of S-100 protein showed thicker bundles, suggesting that the antibody recognized several supportive cells such as glial and Schwann cells (Fig. 8, C and D). RX-77368 induced Fos expression in the corpus (Fig. 8A) and antrum (Fig. 8B) within the network formed by S-100 protein and neurons as observed by double labeling (Fig. 8, E and F, in corpus and antrum, respectively).

Table 2. Effect of peripheral injection of atropine on intracisternal TRH analog RX-77368-induced Fos expression in gastric myenteric cells

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Dose, mg/kg</th>
<th>n</th>
<th>Corpus</th>
<th>Antrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atropine + saline</td>
<td>2</td>
<td>4</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Saline + RX-77368</td>
<td>2</td>
<td>2</td>
<td>17.4 ± 0.7</td>
<td>20.9 ± 0.1</td>
</tr>
<tr>
<td>Atropine + RX-77368</td>
<td>1</td>
<td>3</td>
<td>16.1 ± 2.3</td>
<td>20.4 ± 1.7</td>
</tr>
<tr>
<td>Atropine + RX-77368</td>
<td>2</td>
<td>5</td>
<td>17.2 ± 1.2</td>
<td>23.7 ± 0.7</td>
</tr>
</tbody>
</table>

Values are means ± SE for the no. of Fos-immunoreactive nuclei/myenteric ganglion quantified as in Table 1 legend; n = no. of rats. RX-77368 or saline was injected intracisternally under short enflurane anesthesia 30 min after the subcutaneous injection of saline or atropine. Conscious rats were euthanized 90 min after intracisternal injection. The corpus and antrum were removed and processed for Fos immunohistochemistry using Fos Ab-5. Indirect immunofluorescence staining was used for Fos detection.
DISCUSSION

The present study shows that the TRH analog RX-77368 injected intracisternally at 50 ng induces a robust Fos expression in the myenteric plexus of the gastric corpus and antrum in conscious fasted rats. Fos immunoreactivity examined in the gastric LMMP whole mount preparations was observed within 60 min (26–27 positive cells/ganglion) or 90 min (25–27 positive cells/ganglion) after the RX-77368 injection. The similar number of Fos-immunoreactive cells at both times is consistent with the kinetic of maximum Fos expression in the enteric nervous system that occurred within 60–90 min after various stimulations (11, 53). RX-77368 action is specific because rare Fos-immuno-reactive cells (~1 cell/ganglion) were observed after the intracisternal injection of saline. This is the first demonstration of Fos expression in the myenteric ganglia within the corpus and antrum after centrally mediated activation of the autonomic nervous system in awake rats. Fos expression has been extensively used to map, at the cellular level, stimuli-related activation of neural circuits in the brain (11, 23) and intestine (28, 29, 37, 51) and to a smaller extent in the stomach (52, 53). A previous study (6) in conscious rats established that refeeding after a fasting period or mechanical gastric distension induced intense expression of c-fos mRNA in gastric myenteric neurons, whereas fasted conditions...
alone did not induce c-fos mRNA. We also found no or one Fos-positive cell per ganglion in the gastric myenteric plexus in fasted conscious rats. Because our studies were performed in food-deprived conscious rats, the widespread Fos expression observed in the gastric myenteric ganglia after the intracisternal injection of RX-77368 was not secondary to food intake or gastric distension.

Fos expression in the gastric myenteric plexus in response to intracisternal RX-77368 is likely to represent an activation of myenteric neurons induced by central vagal cholinergic excitatory input on nicotinic ACh receptors. Indeed, TRH exerts a direct postsynaptic increase in the firing rate of DMN neurons (47), and the TRH analog injected intracisternally stimulates gastric vagal efferent discharges in urethane-anesthetized rats (32). Neuroanatomic tracing studies (14) have established the presence of a dense network of vagal efferent terminals encircling and making putative contacts with all myenteric neurons in the rat corpus and antrum. Such distribution of vagal innervation is consistent with the widespread ganglionic Fos expression in both antrum and corpus after the intra-

Fig. 6. Confocal microscope images of gastric LMMP whole mount preparations from rats injected intracisternally with RX-77368 (50 ng) and double labeled with the combination of monoclonal mouse anti-Fos (TF161) and polyclonal rabbit anti-calretinin. RX-77368 triggered Fos expression (arrow) in the myenteric ganglia within the corpus (A and E) and antrum (B and F). Nerve fibers and varicosities as well as a small proportion of nerve cell bodies immunoreactive to calretinin were present in these myenteric ganglia (C and D). Calretinin-immunoreactive nerve fibers surrounded all Fos-immunoreactive nuclei in the corpus (E) and antrum (F). Occasionally, nerve cell bodies immunoreactive to calretinin expressed Fos after intracisternal RX-77368 as observed in the antrum (F, arrow). Note that the images at left (A, C, and E) and right (B, D, and F) were captured on the same double-labeled gastric LMMP whole mount preparation from the corpus or antrum, respectively, by using different filters on a Zeiss LSM laser scanning microscope.

Fig. 7. Confocal microscope images of gastric LMMP whole mount preparations from rats injected intracisternally with RX-77368 and double labeled with the combination of Fos Ab-5 and monoclonal mouse anti-glial fibrillary acidic protein (GFAP). Fos-immunoreactive nuclei (arrows and arrowheads) were present in the myenteric ganglia in both the corpus (A) and antrum (B) after intracisternal injection of RX-77368. GFAP staining showed strands of glial cells (small *) around neurons (large *) in both the corpus (C) and antrum (D). Fos-immunoreactive nuclei observed in A and B after intracisternal RX-77368 were either neurons (arrows) or glial cells (arrowheads) in the corpus (E) and antrum (F). Note that the images at left (A, C, and E) and right (B, D, and F) were captured on the same double-labeled gastric LMMP whole mount preparation from the corpus or antrum, respectively, by using different filters on a Zeiss LSM laser scanning microscope.
with hexamethonium (10–20 mg/kg) dose-dependently reduced the number of Fos-positive cells by 70% and 89%, respectively, whereas atropine had no effect. It is unlikely that hexamethonium acts centrally to interfere with RX-77368 action because TRH directly excites preganglionic motoneurons (47) and microinjection of nicotine into the DMN induced vagal-dependent stimulation of intragastric pressure but was not antagonized by hexamethonium (7). Although the relationship between activation of nicotinic receptors and Fos expression in the central nervous system neurons has been well established in vitro as well as in vivo (10, 11), the present pharmacological and immunohistochemical observations indicate that it is also a feature of the gastric myenteric neurons. Collectively, these findings suggest that the major component of Fos expression in the rat gastric myenteric plexus is mediated by peripheral hexamethonium-sensitive nicotinic receptors. Electrical stimulation of the cervical vagus at 48 Hz for 30 min in anesthetized rats also induced a widespread Fos expression in gastric myenteric neurons, although a larger component of the Fos response was found to be hexamethonium resistant (52, 53).

The experimental approach used in the present study did not allow us to determine whether widespread activation of the gastric myenteric cells in the corpus and antrum occurred mainly through a direct vagal monosynaptic nicotinic process. Previous studies (39) in guinea pig stomach have provided electrophysiological and pharmacological evidence that the fast excitatory postsynaptic potentials in gastric myenteric neurons, in response to electrical vagal stimulation, involve direct monosynaptic nicotinic receptor activation. The demonstration of varicose contacts between vagal fibers and all myenteric neurons in the rat stomach and showed Fos expression in gastric myenteric nerves after electrical stimulation of the cervical vagus except in the denervated area, arguing against interneuronal activation. Whether or not Fos expression observed in the gastric myenteric plexus after intracisternal RX-77368 may also be related to spreading of excitation through second- or higher-order neurons recruited by polysynaptic pathways and/or nicotinic-dependent release of hormones or other transmitters, acting via a paracrine or hormonal mechanism, could not be ruled out in the present study.

In addition to nicotinic mechanisms, other noncholinergic-dependent pathways are involved in intracisternal RX-77369-induced Fos expression in the gastric myenteric plexus. Indeed, 10% of myenteric cells still expressed Fos-immunoreactive in the presence of hexamethonium pretreatment (20 mg/kg sc). Although these results could suggest that hexamethonium injected in such regimen was not sufficient to produce a full blockade of nicotinic receptors, it is more likely that a portion of activated myenteric neurons is driven through second- or higher-order neurons recruited by polysynaptic pathways and/or nicotinic-dependent release of hormones or other transmitters, acting via a paracrine or hormonal mechanism, could not be ruled out in the present study.

In addition to nicotinic mechanisms, other noncholinergic-dependent pathways are involved in intracisternal RX-77369-induced Fos expression in the gastric myenteric plexus. Indeed, 10% of myenteric cells still expressed Fos-immunoreactive in the presence of hexamethonium pretreatment (20 mg/kg sc). Although these results could suggest that hexamethonium injected in such regimen was not sufficient to produce a full blockade of nicotinic receptors, it is more likely that a portion of activated myenteric neurons is driven

---

**Fig. 8.** Confocal microscope images of gastric LMMP whole mount preparations from rats injected intracisternally with RX-77368 and double labeled with the combination of Fos Ab-5 and monoclonal mouse anti-S-100 protein. Fos-immunoreactive nuclei (arrow and arrowhead) were present in the myenteric ganglia in both the corpus (A) and antrum (B) after RX-77368. S-100 protein staining showed bulbous groups of immunolabeled strands (small *) around nonimmunoreactive perikarya, which were neurons (large *) in both the corpus (C) and antrum (D). Fos nuclei observed in A and B after intracisternal RX-77368 were from either neurons (arrow), glial cells, and/or other supportive cells (arrowhead) in the corpus (E) and antrum (F). Note that the images at left (A, C, and E) and right (B, D, and F) were captured on the same double-labeled gastric LMMP whole mount preparation from the corpus or antrum, respectively, by using different filters on a Zeiss LSM laser scanning microscope.
by other nonnicotinic mechanisms. Indeed, hexamethonium at 10 mg/kg injected intravenously completely abolished the vagal cholinergic increase in gastric motility induced by the microinjection of TRH into the DMN (35). Muscarinic receptors are present in the enteric nervous system and mediate excitatory responses in enteric neurons in addition to nicotinic ACh receptors (49). However, blockade of muscarinic receptors with atropine influenced neither Fos expression in the gastric myenteric plexus induced by intracisternal injection of RX-77368 nor the low basal level of Fos expression in rats injected intracisternally with saline. Atropine was administered at doses (1 and 2 mg/kg sc) previously shown (9, 18, 35, 44) to prevent the gastric secretory and motor responses to intracisternal TRH. These results indicate that the muscarinic cholinergic receptors do not contribute to the observed activation of gastric myenteric cells after central RX-77368 either directly at the level of enteric neurons or indirectly through alterations of gastric function. Likewise, atropine was reported not to alter Fos in gastric myenteric neurons in response to electrical stimulation of the cervical vagus nerve (52). Whether the hexamethonium and atropine-resistant component of activated ganglionic myenteric cells after central RX-77368 is related to ATP, serotonin, and/or glutamate, demonstrated to participate in the noncholinergic excitatory transmission in the guinea pig enteric nervous system (8), requires further investigation.

The identity of Fos-positive neurons induced by intracisternal injection of RX-77368 remains largely unknown. Functional studies (9, 18, 26, 43) indicate that gastric myenteric cholinergic motoneurons are activated by TRH or TRH analog injected intracisternally as shown by the stimulation of gastric secretory and motor function through vagal atropine-sensitive mechanisms. We found that all VACHT immunoreactive-labeled myenteric cell bodies were Fos positive, although their total number (20%) most likely underestimates cholinergic postganglionic neurons activated by intracisternal RX-77368. Indeed, VACHT is a marker that is mainly used to label terminal fields of the cholinergic nervous system (Ref. 38 and present study). Other antibodies directed to choline acetyltransferase (ChAT) poorly detect cholinergic cell bodies in the rat peripheral organs even though these antibodies successfully visualize cholinergic neurons in the central nervous system. The recent cloning of a splice variant of ChAT mRNA localized preferentially in peripheral nerve cells and fibers suggests that a different form of ChAT exists in peripheral tissues and may account for these discrepancies (46). Indeed, the antibody raised against the peripheral type of ChAT (pChAT) has revealed that 62% of ganglionic cells present in the muscle layer of the rat stomach are immunoreactive to pChAT (31). Consequently, the percentage of pChAT-immunoreactive myenteric cell bodies expressing Fos after the intracisternal injection of RX-77368 could be different from those reported in the present study. This will require further investigation when the antibody directed against pChAT becomes commercially available.

Calretinin, a calcium-binding protein that is neuron specific, was present in the gastric myenteric ganglia. We found abundant nerve fibers and a small proportion of nerve cell bodies immunoreactive to calretinin in the rat gastric LMMP whole mount preparations. A previous report (16) indicates that calretinin immunoreactivity in the myenteric plexus of rat stomach is present only in fibers and not in cell bodies. It is likely that the discrepancy between the previous study (16) and the present study is due to the differences in the type of gastric preparations used to assess the distribution of calretinin immunoreactivity. Indeed, we labeled cells in gastric LMMP whole mount preparation, whereas frozen cross sections were used in the other study (16). Because calretinin-immunoreactive cell bodies were infrequent in LMMP whole mount preparations, which preserve and display intact myenteric ganglia and interganglionic connective fibers (present study), the labeling of the sparse calretinin-immunoreactive cell bodies may be more difficult to visualize in cross sections of the gastric tissue. The fact that the frequency and distribution of calretinin-immunoreactive nerve fibers and cell bodies observed in our study were similar to those reported (40, 48) in the gastric LMMP whole preparations of guinea pig antrum strengthens our interpretation. Calretinin-immunoreactive nerve fibers were in close contact with Fos-immunoreactive nuclei induced by intracisternal injection of RX-77368. Calretinin functions by binding intracellular free calcium to regulate its concentration and thereby could activate or trigger proteins and indirectly influence biochemical processes that are calcium dependent such as firing patterns in neurons (1). Because calretinin is a calcium-binding protein that could affect calcium flux, the presence of calretinin-immunoreactive fibers around all Fos-positive nuclei may have a significant role in processes that are calcium dependent, such as the firing patterns in neurons. For example, the immediate-early gene c-fos encodes for the nuclear Fos protein after a voltage-gated calcium entry into the cell (5). In guinea pig intestine, calretinin immunoreactivity has been reported (4, 24) to be confined to cholinergic neurons. Because antiserum to VACHT and calretinin used in the present study were both rabbit polyclonal, we were not able to perform a double immunohistochemical labeling to assess the colocalization of calretinin and VACHT as described in guinea pig intestine (4, 24).

The use of Fos Ab-5 with the neuronal marker PGP 9.5 revealed that Fos was expressed in neuronal and nonneuronal cells after intracisternal RX-77368. However, the combination of monoclonal anti-Fos (TF161) with polyclonal anti-PGP 9.5 showed the presence of Fos mainly in gastric myenteric neurons after intracisternal RX-77368. Such differences in Fos labeling may be related to the fact that the polyclonal antibody (Fos Ab-5) binds to several epitopes in the Fos protein, whereas the monoclonal antibody (TF161) binds to one epitope. In glial cells, Fos may interact with another

The use of Fos Ab-5 with the neuronal marker PGP 9.5 revealed that Fos was expressed in neuronal and nonneuronal cells after intracisternal RX-77368. However, the combination of monoclonal anti-Fos (TF161) with polyclonal anti-PGP 9.5 showed the presence of Fos mainly in gastric myenteric neurons after intracisternal RX-77368. Such differences in Fos labeling may be related to the fact that the polyclonal antibody (Fos Ab-5) binds to several epitopes in the Fos protein, whereas the monoclonal antibody (TF161) binds to one epitope. In glial cells, Fos may interact with another
protein so that the epitope is covered and the monoclonal antibody is unable to bind to the Fos protein. Previous electron microscopy studies (9) demonstrated an intimate association between enteric glia and neuronal membranes, with numerous contacts between the two cell types. In addition, enteric glia, as well as neurons, exhibit a neurelloid signal transduction system linked to increased cytosolic calcium levels (17), which are known to be involved in Fos expression (11). Using a double labeling of Fos with S-100 protein, which labeled both glial and Schwann cells (30), we observed that supportive cells display Fos immunoreactivity after intracisternal injection of RX-77368. Double labeling Fos with GFAP, which is a protein expressed exclusively in enteric glia (30), showed that gastric myenteric glia were part of the nonneuronal cells activated by intracisternal RX-77368. The coupling of neuronal and glial cell activation in the gastric myenteric plexus in response to central stimulation has not been reported before. Previously, we (28) observed that neurons as well as nonneuronal cells were activated in the colonic myenteric ganglia after injection of formalin in the rat colonic wall. Little is known about the regulation of glial cell activity in the gastrointestinal enteric nervous system. However, in the central nervous system, there is evidence that many factors released by neurons can elicit early gene expression to coordinate glial and long-term neuronal activities (11). The underlying mechanisms through which glia are activated and their coupling with gastric myenteric neuronal activity deserve further investigation.

Activation of TRH receptors plays a physiological role in acute cold-induced vagal cholinergic stimulation of gastric transit in rats (27). In the present study, the TRH analog RX-77368 was administered at doses eliciting vagal cholinergic-mediated gastric responses similar to those induced by acute cold exposure (26, 27). Therefore, the present data performed in conscious freely moving rats may have relevance to the gastric myenteric activation when medullary TRH pathways are recruited under physiological conditions.

In summary, the TRH analog RX-77368, injected intracisternally at doses known to induce vagal efferent discharges, results in a widespread ganglionic Fos expression in the myenteric neurons within the corpus and antrum of the stomach as shown by double labeling with the specific neuronal marker PGP 9.5. The presence of VACHT around all Fos-positive neurons and the 90% inhibition of Fos expression in gastric myenteric ganglia by hexamethonium, whereas atropine had no effect, indicate that a large proportion of myenteric neurons are activated through nicotinic ACh receptors. In addition, we showed that intracisternal injection of RX-77368 activates glial cells in gastric myenteric ganglia as revealed by double labeling with GFAP and the broader maker of enteric supportive cells, S-100 protein. These results provide new insight into the activation of gastric myenteric cells by central vagal efferent stimulation in conscious rats.

**Perspectives**

The present study demonstrated that central vagal efferent activation induced by intracisternal injection of TRH analog in the fasted conscious rat triggered Fos expression in both gastric myenteric neurons and glia. This constitutes a new model to study vagal input to gastric myenteric ganglia in conscious rats. The vagal excitatory input mediating Fos expression in the gastric myenteric plexus under these conditions involves mainly ACh, which acts on nicotinic cholinergic receptors, whereas muscarinic receptors do not play a role, as indicated by the pharmacological approach. Although the role of nicotinic ACh receptors in ganglionic neurotransmission is well established, the localization and subunit composition of these receptors has received little attention and has been studied exclusively in the guinea pig enteric nervous system (19). Immunohistochemical visualization of nicotinic ACh receptors in Fos-positive cells will be important to provide additional insight into the nicotinic component of the myenteric nervous response. Whether the noncholinergic pathways involved in the hexamethonium-atropine resistant component of activated myenteric cells is mediated by noncholinergic excitatory ganglionic mechanisms as established in the guinea pig enteric nervous system (8) will be worth investigating. RX-77368 injected intracisternally induced widespread Fos expression in the ganglionic myenteric neurons. The classification of activated neurons by function (motorneurons, interneurons, or others) will provide additional information about the myenteric neuronal targets of cholinergic inputs. Additional study to define the biochemical coding of Fos-positive neurons will be required to provide insight into postganglionic excitatory and inhibitory pathways activated by central vagal efferent stimulation in awake animals. The role of activated myenteric glia after central stimulation of efferent vagal pathways also warrants further investigation. Understanding whether gastric myenteric glial cells play only a supportive role or constitute the myenteric ganglionic component that receive cholinergic inputs before the neural component will provide new information on the gastric myenteric neuron and glial cell relationship after central vagal activation.

We thank Dr. T. Waters (Ferring Pharmaceuticals, Feltham, Middlesex, UK) for the generous supply of RX-77368 and Dr. D. R. Scott (CURE: Digestive Diseases Research Center, University of California) for assistance with confocal microscopy. We also thank P. Kirsch for help with manuscript preparation.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-33061 (Y. Tache) and DK-41301 (Animal and Imaging Cores, Y. Tache, G. Sachs). K. A. Sharkey is an Alberta Heritage Foundation for Medical Research Senior Medical Scholar.

**REFERENCES**


2. **Arancibia S, Tapia-Arancibia L, Assenmacher I, and Astier H.** Direct evidence of short-term cold-induced TRH re-

---

**Downloaded from [http://ajpgi.physiology.org/](http://ajpgi.physiology.org/) by 10.220.33.4 on June 24, 2017**


39. Sharkey KA, Parr EJ, and Keenan CM. Immediate-early gene expression in the inferior mesenteric ganglion and colonic


