Galanin Inhibits Gut-Related Vagal Neurons in Rats

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Submitted 5 September 2003; accepted in final form 17 December 2003

Tan, Zhenjun, Ronald Fogel, Chunhui Jiang, and Xueguo Zhang. Galanin inhibits gut-related vagal neurons in rats. J Neurophysiol 91: 2330–2343, 2004. First published December 24, 2003; 10.1152/jn.00869.2003. Galanin plays an important role in the regulation of food intake, energy balance, and body weight. Many galanin-positive fibers as well as galanin-positive neurons were seen in the dorsal vagal complex, suggesting that galanin produces its effects by actions involving vagal neurons. In the present experiment, we used tract-tracing and neurophysiological techniques to evaluate the origin of the galaninergic fibers and the effect of galanin on neurons in the dorsal vagal complex. Our results reveal that the nucleus of the solitary tract is the major source of the galanin terminals in the dorsal vagal complex. In vivo experiments demonstrated that galanin inhibited the majority of gut-related neurons in the dorsal motor nucleus of the vagus. The electrophysiological effects of galanin were mediated by a high-affinity Gi/Go protein-coupled receptor that involved potassium and calcium channels. These effects of galanin are mediated by high-affinity Gi/Go protein-coupled receptors that involve potassium and calcium channels as well as adenylyl cyclase (Pieribone et al. 1998). We postulate that galanin suppresses gut-related DVC neurons through a similar mechanism.

To date, we have limited knowledge regarding the mechanisms by which galanin affects gut-related DVC neurons. In the present experiment, we used double immunohistochemistry and electrophysiological recording techniques to characterize: 1) the source of galanin-containing terminals in the DVC; 2) the effects of galanin on the firing rate of gut-sensitive DMNV neurons; 3) the effects of galanin on DMNV neurons that project to the stomach; and 4) the putative ion channels mediating the galanin effect.

METHODS
The Institutional Animal Care and Use Committee (IACUC) of Henry Ford Health Sciences Center approved all experimental procedures.

RETROGRADE TRACT TRACING AND IMMUNOHISTOCHEMISTRY
Tract tracing and double staining for galanin was used to characterize galanin immunoreactivity in the DVC of 10 adult male Sprague–Dawley rats. Galanin-immunoreactive nerve fibers were observed in the DMNV that project to the stomach; and 4) the putative ion channels mediating the galanin effect.

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Dawley rats. The animals were deeply anesthetized with sodium pentobarbital [50 mg/kg body weight, intraperitoneally (ip)]. The level of anesthesia was assessed by paw pinch reflex and muscle tone. Supplemental doses of sodium pentobarbital (25 mg/kg, ip; total dose <100 mg/kg) were administered if animals responded to paw pinch and increased muscle tone were found. Two surgical procedures were performed.

1) Gastric tracer injection (5 animals). The abdomen was shaved and cleaned with 70% alcohol. The stomach was exposed and 1.0 µl 1% Alexa-Fluor-555-conjugated cholera toxin B subunit (Molecular Probes, Eugene, OR) was injected into the anterior wall of the stomach. Care was taken to prevent leakage into the intestine. The surgical area was cleaned with warm sterile saline solution. The muscle and skin were closed separately with sterile 4-0 silk suture.

2) Brain stem tracer injection (5 animals). Rats were placed in a Kopf stereotaxic frame (David Kopf Instruments, Tujunga, CA). While in the frame, body temperature was maintained at 37°C by a thermostatically controlled heating blanket (custom-made). The brain stem was exposed by removing the atlanto-occipital membrane. We injected 0.2 µl 1% Alexa-Fluor-555-conjugated cholera toxin B subunit into the DVC at the level of the obex (0.5 mm lateral to the obex and 0.5 mm ventral to the dorsal surface of the brain stem). The surgical area was cleaned with warm sterile saline solution. The muscle and skin were closed separately with sterile 4-0 silk suture.

The animals were left on the heating table to recover. Buprenorphine (Reckitt and Colman Pharmaceuticals, Richmond, VA) was administered to reduce postsurgical pain (0.05 mg/kg, ip). Additional doses were given as needed (0.05 mg/kg, ip; twice daily for 2 days). The whole surgical procedure was performed under proper aseptic standards and the animals were maintained in a surgical plane of anesthesia during surgery. After surgery, the animals received routine postsurgical care in accordance with the system guidelines.

To facilitate the histochemical identification of ganalin, we injected 10 µl colchicine (4% in DMSO, Sigma, St. Louis, MO) in the lateral ventricle 1 to 2 wk after tracer injection. Colchicine blocks the axonal transport of ganalin as well as increases mRNA and peptide synthesis in neuronal cells (Cortes et al. 1990). The surgical procedures were the same as those previously stated except that a small burr hole was drilled for injection instead of using open brain stem surgery.

Twenty-four hours after colchicine injection, the animal was deeply anesthetized with sodium pentobarbital (100 mg/kg) and perfused through the heart with 500 ml saline and then 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB). The brain was removed and immersed in 0.1 M PB overnight, and then sliced at 200 µm using a vibrotome. Brain sections were incubated in a solution containing rabbit anti-ganalin (1:200) at 4°C for 7 days, rinsed for 6 h, and incubated in a solution containing fluorescein-labeled goat anti-rabbit IgG (1:50) for 6 days. This long incubation was necessary to allow antibody penetration through the thick tissue sections. The sections were rinsed with 0.4% Triton PB overnight, mounted on a slide, air-dried, and covered with Glycergel (Dako, Carpinteria, CA). The sections were inspected and photographed using a Nikon PCM 2000 confocal microscope (Nikon, Melville, NY).

Electrophysiological recording of gut-related DMNV neurons in vivo

Adult male Sprague–Dawley rats weighing 270–350 g were anesthetized with sodium pentobarbital (50 mg/kg, ip). The anesthetic level was assessed by paw pinch reflex and muscle relaxation. Supplemental doses of sodium pentobarbital were administered as needed to maintain a deep level of anesthesia and muscle relaxation. A tracheotomy was performed and a tube was inserted into the trachea for subsequent artificial ventilation with air and 100% O2 (15 ml/min). A midline abdominal incision was used to expose the abdominal vagus, stomach, and duodenum. Teflon-coated pure gold wire stimulating electrodes (76 µm OD) were placed around the anterior and posterior branches of the subdiaphragmatic vagal nerve. The electrodes were positioned 1–2 cm above the gastroesophageal junction and immediately above the accessory and celiac branches of the vagus nerve. Extra caution was taken not to crush or stretch the nerve. After placing the vagal stimulating electrodes, a gastric influx catheter was inserted into an incision in the gastric corpus and fixed to the greater curvature of the gastric corpus. Two tubes were inserted into the gut through an incision immediately proximal to the pylorus, one oriented toward the stomach and the other oriented toward the duodenum. The tube oriented toward the stomach served as the gastric influx catheter, whereas the tube oriented toward the duodenum was the duodenal influx catheter. The gut was transected 10 cm distal to the ligament of Treitz. The resulting caudal open end was closed with silk suture, whereas the proximal end was cannulated with a tube to serve as the duodenal influx catheter. The abdomen was then closed, and a small piece of gauze was used as a sponge to prevent an accumulation of secretory fluid in the abdomen.

Rats were placed in a Kopf small animal stereotaxic frame after placement of the gastric and duodenal catheters. While in the stereotaxic frame, body temperature was maintained by a thermostatically controlled heating table that also warmed all perfusion fluids to body temperature. The brain stem was exposed by removing the atlanto-occipital membrane and a portion of the occipital bone. A beveled glass micropipette recording electrode (A-M Systems, Everett, WA; 0.10 mm diameter, 0.10 µm resistance = 50–70 MΩ), filled with 1.0 M KCl, was lowered into the vagal complex between 100 µm rostral and 400 µm caudal to the obex where a majority of gut-related neurons are located.

A separate triple-barreled pipette filled with varying concentrations of ganalin and a control solution was placed into the DVC near the recording electrode. The triple-barreled pipette was controlled by motorized micrometer heads (custom-made). This apparatus was able to deliver a volume of the selected solution as small as 1.0 nl by bolus injection or continuous perfusion.

One hour after the surgery, biphasic electrical pulses (duration 1.0 ms, 3–10 V, 1.0 Hz) were delivered to the abdominal vagus as searching stimuli. The recording electrode was advanced until a unit driven by the stimulating electrodes was encountered. The neuron’s response to perfusion of the stomach and duodenum with 0.9% saline (warmed to 37°C) was then determined. Recordings were made while the efflux catheters were in the plane of the animal and again when the efflux catheters were elevated 20 cm above the plane of the animal while continuously perfusing the stomach or intestine (20 ml/min). The change in firing rate in response to any or all stimuli was tested at least twice. If the response was equivocal, the stimulus was presented up to 5 times. Unit discharges were amplified by an A-M Systems high-input-impedance preamplifier and displayed and stored on an IBM-compatible computer with Axoscope software (Axon Instruments, Foster City, CA).

After characterization of the response to gastrointestinal distention, we microinjected galalin into the DVC (the effective dosage and concentration were determined in pilot studies). The neuronal activity was continuously monitored and recorded for off-line analysis. DMNV neurons were identified by retrograde activation after electrical stimulation of the subdiaphragmatic vagus nerve. Retrograde activation criteria included constant latency, ability to follow a high-frequency electrical stimulation of the vagus nerve (75–150 Hz), and a positive collision test. NST neurons were recognized by their anterograde response (variable latency, after low-frequency stimulation, and multiple responses to a single vagal stimulus). If a neuron did not show specific properties of DMNV or NST neurons, the cell was excluded from analysis.

Recording retrogradely labeled DMNV in vitro

Sprague–Dawley rats (10–20 days old, 20–40 g body weight) were deeply anesthetized with sodium pentobarbital (50 mg/kg body weight, ip). The abdomen was shaved and cleaned with 70% alcohol.
The stomach was exposed by a midline incision of the abdomen. Dil solution (3% in DMSO, 0.1 μl; Molecular Probes) was intramuscularly injected into the anterior antrum using a 36-gauge microsyringe. The surgical area was then cleaned with warm sterile saline solution, the excess solution blotted with sterile cotton tips, and the muscle and skin were closed separately with sterile 6-0 silk suture. The animal was left on the heating table to recover. If dehydration was observed, we injected 1–2 ml sterile saline intraperitoneally to compensate for lost fluid. After the animal recovered from anesthesia, one dose of Buprenex (0.05 mg/kg) was injected intraperitoneally to reduce post-surgical pain. Additional Buprenex was given as needed. The animal was returned to the cage and allowed free access to the mother’s milk. The entire surgical procedure was performed under aseptic standards and the animals were maintained in a surgical level of anesthesia during surgery. After 4–7 days, the rat was deeply anesthetized and killed. The brain was quickly removed and placed for 2–5 min in an oxygenated storage chamber containing ice-cold physiological rat artificial cerebral spinal fluid (ACSF: 95% O₂ and 5% CO₂) to harden before the cutting procedures. A block of the brain including the DVC was cut and glued to a plastic support where 2 to 4 slices (400 μm thick) were cut along the coronal plane using a vibrotome. The brain slices were collected into a container, incubated, and equilibrated at 32°C in an oxygenated, physiological rat ACSF for at least 1 h. Thereafter, a single slice was mounted on a custom-made perfusion chamber and anchored in place. The chamber was continuously perfused with the oxygenated ACSF at 3.0 ml/min.

Recordings of the labeled neurons were performed using an epifluorescent in vitro recording microscope (Nikon Eclipse E600-FN, Nikon, Tokyo, Japan). The DMNV neuron labeled by the retrograde tracer was approached with a patch pipette filled with the intracellular solution, with a tip resistance of 7–10 MΩ. Before immersion of the pipette into the perfusion solution, a positive pressure was applied to the pipette to prevent obstruction. At the same time, the pipette was balanced and compensated for resistance and capacity in the bridge mode. When a fluorescent-labeled DMNV neuron was identified, the patch pipette was advanced to touch the cell using a 3-dimensional micromanipulator. The resistance of the electrode was continuously monitored. When the pipette touched the cell, as indicated by an increased resistance and a dent on the neuron, a negative pressure was applied to the pipette while the seal resistance was continuously monitored. When the seal resistance increased to 1 GΩ or more, indicating formation of the seal, a slight increase or a pulse of negative pressure was used to break the cell membrane and whole cell recording was established. After the intracellular membrane potential had been established, the neuron was clamped to a hyperpolarizing level of −60 mV to inhibit the spontaneous current activities and to avoid toxic calcium loading in the neuron. The voltage was gradually reduced until the normal resting membrane potential (−40 to −55 mV) and then the recording experiment began.

Neurons having resting potentials < −50 mV, action potentials > 60 mV, overshooting > 5 mV, and membrane resistance > 100 MΩ (indicating the neurons are healthy) were further characterized for their response to galanin. Neuronal activity was recorded at 5 kHz using an Axoclamp 2B microelectrode amplifier (Axon Instruments, Foster City, CA), digitized using Digidata 1322 (Axon Instruments), and stored on a computer using the Axoclamp software for later statistical analysis. The liquid junction potential was corrected according to the ion contents using a conventional formula (Axon Instruments).

Drugs were applied using a slow-infusion technique through a separate tube calibrated by electronically operated valves (ALA-VM8, ALA Scientific Instruments, New York, NY) and driven by gravity. The distance from the tip of the drug-delivery tube to the recorded neurons was 2–3 mm. The delivered drug was diluted by the chamber ACSF solution; thus the final concentration of the drug at the neuron was unknown. Pretreatment with antagonists and ion substitution experiments were conducted by replacing normal ACSF solution with the selected solution using manually controlled valves.

Data analysis

For the spontaneous activities of the neurons both in vivo and vitro experiments, we took a 30-s trace before application of galanin and a 30-s trace after application of galanin. The timing of the 30-s trace after galanin depended on the experiments. A time frame of showing the maximum effect was chosen (1–10 min after galanin) during each experiment because galanin’s latency is different for each neuron. Each 30-s trace was divided into 6 bins (5 s/bin). The traces before and after galanin were compared using Student’s t-test. To determine whether the change in firing rate was significant in a group of neurons, we compared the total number of action potentials before and after galanin administration using paired t-test. All data are expressed as means ± SE. Changes were significant only if the firing rate after galanin was statistically different from that before galanin (P ≤ 0.05).

The analysis of the responses to gastrointestinal distention in the in vivo experiment was identical to that previously used (Fogel et al. 1996). A 120-s trace of recorded neuronal activities was divided into 4 periods (30 s each) to test the effect of each stimulus. Period 1 represented basal spontaneous activity. Period 2 included the immediate response to the stimulus, whereas period 3 represented the late response to the stimulus. Period 4 contained the first 30 s after the stimulus was discontinued and therefore represented any delayed responses or changes induced by removal of the stimulus. Bins of 5 s (total action potentials in 5 s) were used to generate a histogram and were evaluated using ANOVA with a post hoc Bonferroni’s test for multiple groups using SPSS software (SPSS, Chicago, IL). Changes were considered significant only if period 2 and/or period 3 were statistically different from period 1 (P ≤ 0.05).

Changes of transmembrane potential and transmembrane current in the in vitro experiments were actual numbers. To be considered significant, they must be greater than the baseline and repeatable.

Solutions and chemicals

ACSF (mM): 125 NaCl, 3.0 KCl, 2.5 CaCl₂, 1.5 MgCl₂, 10 dextrose, 25 NaHCO₃ (pH 7.4) saturated with 95% O₂ and 5% CO₂ (modified for brain slice recording). Intracellular solution (mM): 128 potassium gluconate, 10 KCl, 0.3 CaCl₂, 1.0 MgCl₂, 10 HEPES, 1.0 EGTA, 2 ATP, 0.25 GTP (pH 7.35 adjusted with KOH). Intracellular solution with Neurobiotin (mM): 135 potassium gluconate, 0.3 CaCl₂, 1.0 MgCl₂, 10 HEPES, 1.0 EGTA, 2.0 ATP, 0.25 GTP, 0.15% Neurobiotin (pH 7.35 adjusted with KOH).

Porcine galanin (Bachem California, Torrance, CA) was dissolved in ACSF as a stock solution and kept under −20°C. It was further diluted to the final solution with fresh ACSF. DiI (Molecular Probes) was dissolved in DMSO and kept in the dark. Tetrodotoxin (TTX), tetraethylammonium (TEA), and other chemicals (Sigma–Aldrich, St. Louis, MO) were prepared before use in ACSF saturated with 95% O₂ and 5% CO₂.

Antibodies, including rabbit anti-galanin antibody (Peninsula Laboratories, San Carlos, CA); rabbit anti-tyrosine hydroxylase (Chemicon International, Temecula, CA); and goat anti-rabbit IgG fluorescence-labeled antibody (Chemicon International), were diluted in 0.1 M PB (pH 7.4) containing 0.4% Triton 100 (Sigma–Aldrich).

RESULTS

Galaninergic fibers innervate NST and stomach-projecting DMNV neurons

After injection of Alexa-Fluor-555-conjugated cholera toxin B subunit into the anterior wall of the stomach, many retrogradely labeled neurons were seen at all levels of the DMNV,
with the heaviest labeling noted in the middle columns. The dendrites of the labeled DMNV neurons extended into the NST (red cells in Fig. 1).

Staining for galanin reveals heavy galanin-positive fibers throughout the DVC, especially in the DMNV and medial subnuclei of the NST (mNST). Labeling was heaviest around or caudal to the obex in both the NST and DMNV. In the rostral part of the DVC, only light to moderate galanin-positive fibers were seen (Fig. 1, F and F'). Many stomach-projecting DMNV neurons were surrounded by heavy galanin-positive fibers (yellow dots).

Numerous galanin-containing neurons were also seen in the mNST at the level of the obex and caudal to the obex (Fig. 1, B' and C'). These cells had small- to medium-size cell bodies. The rostral NST had only a few neurons. No double-labeled DMNV neurons were found.

The NST is the major source of galaninergic fibers in the DVC

In a different group of rats, we determined whether neurons projecting to the DVC contained galanin. We injected Alexa-Fluor-555-conjugated cholera toxin B subunit into the DVC to identity neurons projecting to the DVC and stained for galanin. Many retrogradely labeled neurons were seen in the PVN (Fig. 2, E and F), the lateral hypothalamus (LH; Fig. 2C), and the CNA (Fig. 2D). Few retrogradely labeled neurons were seen in the LC and the area ventral to the LC.

Numerous galanin-containing neurons and fibers in the LC and the PVN were seen (Fig. 2, A and B). There were moderate galanin-positive fibers in the LH (Fig. 2C) and the CNA (Fig. 2D). The majority of the galanin-positive neurons in the amygdala were located in its medial nucleus and few galanin-positive neurons were seen in the lateral and the anterior amygdaloid nuclei (AAN) and the central amygdaloid nucleus (CAn).}

FIG. 1. Photographs are single sections of a confocal series from 200-μm-thick tissues. Only contrast and brightness were adjusted. Red cells were dorsal motor nucleus of vagus nerve (DMNV) neurons labeled after injection of the retrograde tracer into the stomach and the green structures were galanin-positive fibers and neurons. Yellow dots (double stained for green and red colors) indicate that the galanin-positive fibers directly synapse on DMNV neurons. Galanin-positive neurons were mainly seen at the middle and the caudal levels of the nucleus of the solitary tract (NST). Relative location of the brain levels of the photographs to the obex is marked at the top right corner. AP, area postrema; C, central canal; IV, fourth ventricle; gNST, gelatinous subnucleus of the nucleus of the solitary tract (NST); mNST, medial subnucleus of the NST; rNST, rostral NST; sNST, subpostremal subnucleus of the NST.
positive neurons were located in the CNA (Fig. 2D). No double-labeled neurons for either the retrograde tracer or galanin were found in the PVN, the LH, the CNA, or the LC (Fig. 2, A–F). In an area surrounding the rostral LC, there were scattered double-labeled fibers (yellow) for both galanin and the efferent terminals from the NST, indicating that galanin-containing neurons in the NST project to this area (Fig. 2B). Some anterograde-labeled fibers from the NST were galanin positive (yellow dots). Moderate galanin-positive fibers terminated around the neurons that projected to the DVC in the lateral hypothalamus (LH) (C) and in the central nucleus of amygdala (CNA) (D). Medial nucleus of amygdala (MNA) had a few galanin-positive neurons (D). Very heavy galanin-positive terminals and numerous galanin-positive neurons were seen in the paraventricular hypothalamic nucleus (PVN) surrounding the PVN neurons that project to the DVC in both the magnocellular PVN (mPVN) and the parvocellular PVN (pPVN; E and F). No double-labeled neurons were found in these areas. Yellow dots in C–F indicate where the galanin-positive fibers contact the cell body and/or dendrites of the retrogradely labeled neurons. Photos are single sections of confocal series from 200 μm thickness of brain sections except E, which is a composite of a confocal series.

Galanin affects gut-related DVC neurons in vivo

In our in vivo experiments, we characterized the response of 28 DMNV neurons to galanin and gastrointestinal distention. Twenty DMNV neurons were inhibited by galanin (80 pmol infused into the DVC). The mean spontaneous activity of the DMNV neurons was 1.14 ± 0.17 Hz (mean ± SE) that was significantly reduced to 0.47 ± 0.15 Hz (a 41% reduction after...
galanin administration, $P < 0.01$; Fig. 3A). The latency varied from 1 to 10 min after galanin injection. The effect of galanin lasted from 1 to 60 min. Galanin excited 3 neurons and did not affect 5 neurons.

Gastric distention inhibited 21 neurons, excited one neuron, and did not affect the remaining 6 DMNV neurons. Intestinal distention inhibited 14, excited 3, and did not affect the remaining 11 DMNV neurons. The effects of galanin and gastrointestinal distention are summarized in Table 1. Representative examples of DMNV neurons that were inhibited by galanin, gastric, and intestinal distention are shown in Fig. 3, C–H.

We also characterized the responses of 8 NST neurons to galanin and gastrointestinal distention. In contrast to the inhibitory effect of galanin on the DMNV neurons, the effect of galanin on the mean basal activity was not significant (Fig. 3B). The mean basal activity of 8 NST neurons was $1.86 \pm 0.36$ Hz. This was suppressed to $1.78 \pm 0.36$ Hz (an 8% reduction, $P > 0.05$). However, among the 8 NST neurons, galanin significantly inhibited 3 and significantly excited 3 neurons. The observation suggests that there are at least 2 sets of NST neurons responsive to galanin (one excited and the other inhibited).

**Galanin inhibited stomach-projecting DMNV neuron in vitro**

We injected DiI into the anterior stomach wall to label DMNV neurons projecting to the stomach. The injection site
TABLE 1. Responses of DMNV neurons to galanin and gastrointestinal distention

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<tr>
<th>Test</th>
<th>Gastric Distention</th>
<th>Intestinal Distention</th>
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<tr>
<td>Galanin</td>
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<td>(+)</td>
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<tr>
<td>(−)</td>
<td>20</td>
<td>15</td>
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<td>(0)</td>
<td>5</td>
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The majority of DMNV neurons that were inhibited by galanin were also inhibited by gastric and intestinal distention. (−): inhibited by the stimulus; (+): excited by the stimulus; (0): not affected by the stimulus.

was inspected using a fluorescent microscope. The tracer was not limited to the antrum but diffused along the length of stomach between the muscle layers. The tracer was not seen in either the small intestine or the colon. Therefore the labeled neurons observed in the DVC projected only to the stomach.

A total of 96 DMNV neurons labeled with DiI were characterized. Fifty-two neurons were spontaneously active and 44 neurons had no spontaneous firing during 1 min of recording.

Responses of DMNV neurons to galanin depended on the concentration of galanin applied. Low doses of galanin (0.1–0.3 μM) inhibited spontaneous firing with or without slight hyperpolarization. Higher doses of galanin (1–10 μM) abolished the spontaneous firing of the neurons and evoked significant hyperpolarization (Fig. 4, A–E). The concentration–response curve to galanin had an EC50 of 1.35 μM (Fig. 4F).

The mean response of 20 spontaneous active DMNV neurons to 10 μM galanin was evaluated. The mean spontaneous firing rate of these 20 DMNV neurons was 1.95 ± 0.51 Hz, significantly higher than that of DMNV neurons recorded in vivo (1.14 ± 0.17 Hz, P < 0.001). Application of 10 μM galanin for 30 s reduced the mean basal firing rate to 0.57 ± 0.48 Hz (P < 0.001). The basal activities of 16 of the 20 DMNV neurons were reduced (3 neurons) or completely abolished (13 neurons.) by 10 μM galanin.

In addition to 10 μM galanin, we also tested the responses of DMNV neurons to 0.1, 0.3, 1, and 3 μM concentrations of galanin. Responses of the other 61 neurons to different concentrations of galanin are summarized in Table 2. Galanin at 1, 3, and 10 μM significantly reduced the mean basal activity of DMNV neurons, whereas 0.1 and 0.3 μM galanin had no significant effects on the mean activity of the DMNV neurons (P > 0.05).

The mean resting membrane potential of 40 DMNV neurons tested with 10 μM galanin was −59.30 ± 1.96 mV (−14.8 mV of junction potential calculated from the conventional formula was included). This mean potential was significantly reduced to −70.85 ± 2.55 mV (−11.55 ± 1.24 mV net change; P < 0.001). The maximum hyperpolarization induced by 10 μM galanin was 32 mV. Among the 40 DMNV neurons, 20 neurons were spontaneously active and 20 neurons were silent. The latency period ranged from 5 to 30 s. The galanin-induced hyperpolarization was reversible. The duration of inhibition varied greatly from 45 to 1,000 s (267.25 ± 39.76 s).

We recorded 12 neurons in voltage-clamp mode. At the hyperpolarization of −55 mV, galanin induced a reversible outward current of 89.08 ± 14.31 pA. The amplitude ranged from 30 to 180 pA in the presence of 10 μM galanin. The

amplitude of the outward current was concentration dependent. An example is shown in Fig. 5.

Galanin inhibited the DMNV neurons postsynaptically

In a separate experiment, we tested the effects of galanin on 7 DMNV neurons in a low–calcium and high–magnesium solution containing TTX (2 μM TTX, 0.25 mM Ca2+, 10 mM Mg2+). This solution was able to differentiate pre- from postsynaptic effects. Galanin (10 μM) induced hyperpolarization (∼21.71 ± 3.22 mV) in normal ACSF. ACSF containing TTX, low calcium, and high magnesium suppressed the spontaneous activity of those neurons that were spontaneously active. Five minutes after the perfusion solution was switched to ACSF containing TTX and low calcium, the hyperpolarization evoked by galanin remained almost unchanged (∼22.43 ± 3.16 mV). The result indicated a postsynaptic effect of galanin on DMNV neurons (Fig. 6).

Galanin inhibited the DMNV neurons through TEA-sensitive potassium channel

The current–voltage relationship in the presence of galanin was plotted initially using standard ACSF (3 mM potassium) solution for 10 DMNV neurons. The reversal potential was −105 mV (at the point the outward current shifted to an inward current). This reversal potential was close to the potassium-ion value calculated from the Nernst equation (Fig. 7).

We studied the galanin response in 11 neurons before and after application of the potassium-channel blocker TEA. The inhibitory response of the DMNV neurons to galanin was either blocked or attenuated after changing the normal ACSF perfusion solution to ACSF containing TEA (3–10 mM, 3–10 min). The effect of TEA was reversible (Fig. 8). The duration and shape of the action potentials were slightly widened after TEA application.

DISCUSSION

Data from the retrograde tract-tracing and immunohistochemistry experiments revealed that 1) the mNST contained numerous galanin-positive neurons; 2) both the DMNV and the NST had dense galanin-positive terminals; 3) DMNV neurons that projected to the stomach were in close proximity to galanin-positive terminals, suggesting a synaptic structure; and 4) the galanin-positive terminals in the DMNV and the NST originated from neurons in the NST, not from other galanin-containing regions known to project to the DVC (the PVN, the LH, the CNA, or the LC).

The in vivo experiments demonstrated that galanin inhibited the majority of DMNV neurons projecting to the gastrointestinal tract. Both inhibitory and excitatory effects of galanin on gut-related NST neurons were observed.

The in vitro experiments confirm our in vivo finding that galanin inhibited the firing rate of the majority of stomach-projecting DMNV neurons. This reversible dose-dependent effect was attributed to membrane hyperpolarization and increased outward current. Galanin acted directly on DMNV neurons, as evidenced by the persistent effect after synaptic input was blocked. The reversal potential induced by galanin was close to the potassium ion potential from the Nernst equation, suggesting that the inhibitory effect of galanin is
mediated by the potassium channel. This potassium channel was TEA sensitive.

Retrograde tract tracing and immunohistochemistry

We used Alexa-Fluor-555-conjugated cholera toxin B subunit and immunohistochemistry to identify 1) the galanin terminals on DMNV neurons that project to the stomach and 2) the source of galaninergic fibers in the DVC. Alexa-Fluor-555-conjugated cholera toxin B subunit was used because the cholera toxin B subunit strongly induces active, receptor-mediated uptake mechanisms (van der Want et al. 1997; Vercelli et al. 2000) and remains visible after prolonged incubation in

FIG. 4. A–E: examples of dose-dependent responses of a DMNV neuron to galanin in the in vitro recording. Duration and concentration of galanin are marked at the top of each trace. F: concentration–response curve for galanin-evoked hyperpolarization relative to 10 μM galanin. Numbers of neurons tested were marked on each test point represented by mean ± SE. EC₅₀ = 1.35 μM.
thick brain slices (200 μm). The prolonged incubation used in this study (6–7 days) was necessary because of the thick brain sections studied in these experiments. The 200-μm sections were chosen to enhance visualization of neuronal structures and the trajectory of the fibers. Finally, colchicine was used to facilitate the detection of galanin immunoreactivity. Colchicine inhibits neuropeptide movement, allowing for visualization of cell bodies. Colchicine also has other effects, such as increasing mRNA expression and peptide synthesis (Cortes et al. 1990).

In the present experiment, we demonstrated numerous galanin-positive structures in the DVC. The galanin-positive neurons were mainly located in the mNST at the level of the

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<th>Response</th>
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<td>0.1</td>
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<tr>
<td>Completely inhibited</td>
<td>0</td>
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<tr>
<td>Incompletely inhibited</td>
<td>5</td>
</tr>
<tr>
<td>Excited</td>
<td>1</td>
</tr>
<tr>
<td>Not affected</td>
<td>4</td>
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<tr>
<td>Total neurons tested</td>
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FIG. 5. Illustration of dose-dependent outward currents evoked by different concentrations of galanin in a DMNV neuron while the membrane potential was held at $-55 \text{ mV}$.
obex and caudal to the obex. The rostral NST contained few galanin-positive neurons. These results are in accordance with the published literature (Gonzalez et al. 1995; Melander et al. 1986). In the DMNV, galanin-containing fibers surrounded DMNV neurons that projected to the stomach.

Similarly to others (Berk and Smith 1994; Melander et al. 1986), we demonstrated galanin-positive neurons and/or fibers in the PVN, the CNA, and the LH, regions known to project to the DVC. None of the galanin-positive neurons in these areas was retrogradely labeled by DVC injection of Alexa-Fluor-555-conjugated cholera toxin B subunit, indicating that the galaninergic neurons in these areas do not project to the DVC. The galaninergic NST neurons are the major source of galanin-positive terminals in the DMNV.

Galanin immunoreactivity has been found in the gastrointestinal tract and in the vagal primary afferent neurons (Calin-gasan and Ritter 1992). We do not believe that the peripheral galanin-containing afferent fibers are the major source of the galaninergic fibers to the DMNV. The heavy labeling of galanin-positive fibers that we observed exceeds what would reasonably be expected from the few vagal primary afferents that terminate in the DMNV (Altschuler et al. 1989; Rinaman et al. 1989).

**Fig. 6.** Application of 10 μM galanin abolished the spontaneous activity and produced a reversible hyperpolarization in a DMNV neuron (A). Replacing the normal artificial cerebrospinal fluid (ACSF) solution with an ACSF solution containing tetrodotoxin (TTX), low calcium, and high magnesium prevented the spontaneous firing, indicating the sodium channel and synaptic activity were blocked (B). However, the hyperpolarization induced by galanin was not affected by the ACSF solution containing TTX, low calcium, and high magnesium (B), suggesting galanin acts on postsynaptic receptors.

**Effect of galanin on gut-related DVC neurons in vivo**

Our in vivo data demonstrated that galanin inhibited the firing rate of the majority of gut-related DMNV neurons. We suggest that this inhibitory effect of galanin is attributable to the direct effect on DMNV neurons (see the in vitro results below).

We noted that the duration of the galanin effect varied greatly in both our in vivo and in vitro experiments (duration of effect ranged from 30 s to 15 min). The distance between the recorded neuron and the infusion pipette in vivo was not constant. It is possible that the difference in the concentration of galanin at the receptors is one explanation for this finding. However, because a similar variation was noted in vitro where the diffusion distance was constant, we suggest that the variation of the galanin effect reflects a nonuniform distribution of galanin receptors on DMNV neurons. Data have shown that gut-related DMNV neurons are heterogeneous for morphology, physiology, and phenotype (Armstrong et al. 1990; Fogel et al. 1996; Kitahama et al. 1992; Willing and Berthoud 1997). Galanin may have a stronger influence on some subgroups of DMNV neurons.

We found that approximately 10% DMNV neurons were slightly excited by galanin. We do not know whether this is a
When the membrane potentials were clamped at or below \(-0.1 \, \text{V}\) to the predicted potassium-ion reverse potential of \(E_K = -102 \, \text{mV}\) from the Nernst equation, indicating galanin activates potassium channels.

direct or indirect effect. NST neurons project to the DMNV and have been shown to inhibit DMNV neurons. Inhibition of the inhibitory NST neurons results in excitation of DMNV neurons (Hornby 201; Zhang and Fogel 2003; Zhang et al. 1995).

Our in vivo experiment demonstrated that galanin excited some NST neurons and inhibited others, indicating heterogeneity of NST neurons (Zhang et al. 1995). This excitatory response is consistent with the finding that injection of galanin induced c-fos expression of a subset of NST neurons (Marcos et al. 2001). Our data do not permit us to comment regarding the relative frequency of each subset. Further work will be needed to clarify the influence of galanin on NST neurons.

We did not test the effect of gastrointestinal distention after injection of galanin. Because the predominant effects of galanin and gastrointestinal distention are inhibition of firing rate of DMNV neurons, we could not produce meaningful data after the basal activity was completely abolished by galanin.

**Galanin inhibited the stomach-projecting DMNV neurons in vitro**

We used DiI to retrogradely label DMNV neurons that projected to the stomach. DiI was observed in DMNV neurons by 24 h after injection and remained visible for several weeks after injection. The fluorescent exposure to identify DiI-containing cells does not affect neuronal function (Honig and Hume 1989; Katz et al. 1984).

In our in vitro experiments, we demonstrated that the majority of stomach-projecting DMNV neurons were inhibited by galanin. Galanin produced a fully reversible dose-dependent membrane hyperpolarization. The galanin-induced hyperpolarization could not be completely reproduced immediately after the spontaneous activity and/or resting membrane potential returned to the prestimulus levels. However, the full galanin-induced hyperpolarization could be observed 10–20 min after the spontaneous activity and/or resting membrane potential returned to the prestimulus levels. A similar desensitization of neurons to galanin has been observed in the dorsal raphe neurons (Xu et al. 1998) and the LC neurons (Pieribone et al. 1995).

The galanin-induced hyperpolarization was mediated by increased membrane permeability for potassium ions (outward current) by a postsynaptic mechanism. This argument is supported by the following evidence: 1) galanin-containing fibers form putative synapses on DMNV neurons; 2) the galanin-induced current was reversed at a potential that is very close to the reversal potential of potassium ions; 3) the galanin-induced hyperpolarization was attenuated by the potassium channel blocker TEA; and 4) the galanin-induced hyperpolarization was not affected in the presence of TTX, low calcium, and high magnesium. The effect of galanin on DMNV neurons was similar to that seen in LC neurons (Pieribone et al. 1995; Xu et al. 1998), parasympathetic neurons (Konopka et al. 1992; Parsons et al. 1998), myenteric neurons (Ren et al. 2001; Tamura et al. 1988), magnocellular neurons in the PVN (Papas and Bourque 1997), and histaminergic tuberomammillary neurons (Schonrock et al. 1991).

In addition to potassium ions, it has been shown that calcium ions are also involved in the mechanism of galanin’s effect on myenteric neurons (Ren et al. 2001), parasympathetic neurons (Parsons et al. 1998), pituitary cells (La Porta et al. 1992), and hippocampal neurons (Palazzi et al. 1991). It is unlikely, however, that calcium ion plays a significant role in galanin’s effect on DMNV neurons, given that low-calcium ACSF had little influence on the hyperpolarization induced by galanin.

The resting membrane potential was \(-59.30 \pm 1.96 \, \text{mV}\), similar to that reported by Sah (1992). The hyperpolarization effect of galanin was similar to but stronger than that seen on LC and dorsal raphe neurons (Pieribone et al. 1995; Seutin et al. 1989; Xu et al. 1998). The concentration–response curve to galanin had an \(EC_{50}\) of 1.35 \(\mu\text{M}\), higher than the 0.6 \(\mu\text{M}\) seen in dorsal raphe neurons (Xu et al. 1998). This difference may reflect methodological factors related to galanin application to reduce the artificial mechanical and/or chemical influences with drug administration, the galanin solution was added in front of the recorded neurons through a separate pipette while the brain slice was continuously perfused with normal ACSF. The applied galanin solution would be diluted on arrival at the recorded neurons by the surrounding ACSF. It is impossible to predict the extent of this dilution. Alternatively, either DMNV neurons may have a different density of galanin receptors or the receptors have a different affinity than that of LC neurons.

Although galanin inhibited most DMNV neurons by a postsynaptic mechanism, presynaptic effects of galanin cannot be ruled out. Investigators have shown that galanin acts by several subtypes of presynaptic receptors to depress synaptic transmission in the rat arcuate nucleus (Kinney et al. 1998) and to inhibit dopamine (Katz and Iarovici 1990) and histamine releases (Arrang et al. 1991). We observed that several DMNV neurons were slightly excited by low concentrations of galanin. Whether this is a direct or an indirect effect is unclear.
galanin effect on NST neurons could result in an excitation of DMNV neurons because NST has a tonic inhibitory influence on DMNV neurons.

In the in vitro recording, the spontaneously active DMNV neurons fired at a regular frequency, consistent with our in vivo observation. However, the mean spontaneous activity in the brain slice was significantly higher than that seen in vivo. This higher basal activity might reflect an excitation caused by the interruption of inhibitory input from NST neurons or from higher brain centers such as the PVN. We previously reported that NST neurons are excited by input from vagal primary afferents. NST neurons inhibit the gut-related DMNV neurons (Hornby 2001; Sivarao et al. 1998). In the brain slice experiments, there is no vagal afferent input to excite NST neurons. A decreased activity of NST neurons could remove the tonic inhibitory effects on DMNV neurons. Alternatively, the higher mean spontaneous firing rate of DMNV neurons in vitro may be attributable to an elimination of the inhibitory input from the

FIG. 8. Drawings show that potassium channels were responsible for the galanin-induced hyperpolarization. Galanin induced a hyperpolarization and prevented spontaneous firing of a DMNV neuron (A). Application of potassium-channel blocker TEA significantly attenuated the effect of galanin (B). After a 30-min wash with the normal ACSF solution, the response to galanin was completely recovered.
PVN. It has been shown that lesions of the PVN increased insulin secretion (Sims and Lorden 1986), indicating a higher vagal activity. Finally, it is possible that use of anesthesia was responsible for the lower firing rate in vivo. Sodium pentobarbital used in vivo has a GABAergic agonist effect on neurons that may contribute to the lower basal firing rate.

We do not know the subtype of galanin receptor(s) responsible for the effect of galanin. Three subtypes of galanin receptors (GalR1, GalR2, and GalR3) have been identified in the CNS and may participate in the effects of galanin (Floren et al. 2000; Gundlach 2004). Both GalR1 (Gustafson et al. 1996) and GalR2 (Depczynski et al. 1998) have been found in the DVC. Injection of M40 (which has a high affinity for GalR1) into the NST attenuated the feeding effect of galanin (Koegler et al. 1999), indicating the involvement of GalR1 in DVC neurons’ response to galanin.

Our results suggest that the mechanism of the DMNV neurons’ response to galanin is similar to that of LC neurons. The hyperpolarization of LC neurons is mediated by GalR1 (Ma et al. 2001), further suggesting that GalR1 is the responsible subtype of galanin receptors in the DVC. GalR2 was originally demonstrated only in the anterior pituitary and hypothalamus (Wynick et al. 1993). However, M15, a high-affinity chimeric peptide for GalR2, blocked the neuronal actions of galanin or displaces the binding sites in the hippocampus, locus coeruleus, and spinal cord, indicating a wide distribution of the GalR2 in the CNS (Bartfai et al. 1991; Depczynski et al. 1998). Whether GalR2 participate in the effect of galanin on DMNV neurons is unknown.

Physiological implications

We demonstrated that galanin inhibited the majority of the gut-related DMNV neurons both in vivo and in vitro. Galanin has been involved in regulation of feeding and gastric motility (Davison and Mah 1997; Koegler et al. 1999; Nagase 2002). The direct inhibition of DMNV neurons by galanin may be one of the mechanisms for the galanin effect on gastrointestinal function.

The mNST receives information from vagal primary afferents from the stomach and the intestine (Altschuler et al. 1989; Zhang et al. 1995, 2000). Heavy galanin-positive terminals in the mNST indicate that galanin in the DVC is involved in the vago-vagal reflex of the gastrointestinal tract.

The vago-vagal reflex induced by gastric distention differs from that induced by intestinal distention. Gastric distention induced a fast response in DMNV neurons with a fast adaptation that could be repeated within several minutes. However, intestinal distention elicited a slower response without adaptation that could last for several minutes (Zhang et al. 2002). The inhibitory response of DMNV neurons to gastrointestinal distention is mediated by NST neurons that may release GABA as a neurotransmitter (Feng et al. 1990; Sivarao et al. 1998; Turski et al. 1990).

In addition to GABA, galanin may play an important role in the gastrointestinal vago-vagal reflex. Because the response of DMNV neurons to galanin was slow and lasted for a long time with desensitization, galanin may not be the major neurotransmitter in the inhibitory response of DMNV neurons to gastric distention. Galanin may play some roles in the inhibitory response of DMNV neurons to intestinal distention because intestinal distention induced delayed and long-lasting inhibition on DMNV neurons. However, further experiments are needed to elucidate this hypothesis.

Data have shown that the DVC is a primary target of galanin’s effect on feeding (Koegler and Ritter 1998). Galanin may inhibit NST neurons that receive satiety signals from the gut, as indicated by the many galanin-positive terminals in the mNST. Blocking of the satiety signals will subsequently increase feeding and body weight (Nagase 2002).

In summary, the present results demonstrated that the heavy galanin-positive neurons and terminals were present in the DVC. The results suggest that galanin-positive terminals in the DVC were from the NST. Galanin inhibited the gut-related DMNV neurons both in vivo and in vitro. Galanin inhibited DMNV neurons by increased permeability of TEA-sensitive potassium channels through a postsynaptic mechanism. The effect of galanin on the DVC neurons may be one of the underlying mechanisms of galanin’s role in gastrointestinal function and feeding regulation.

References


