Glucose does not activate nonadrenergic, noncholinergic inhibitory neurons in the rat stomach

Min Shi,1 Allison R. Jones,2 Manuel Ferreira, Jr,2 Niaz Sahibzada,2 Richard A. Gillis,2 and Joseph G. Verbalis1

Departments of 1Medicine and 2Pharmacology, Georgetown University Medical Center, Washington, DC

Submitted 8 August 2004; accepted in final form 1 November 2004

Stromal glucose does not activate nonadrenergic, noncholinergic inhibitory neurons in the rat stomach. Am J Physiol Regul Integr Comp Physiol 288: R742–R750, 2005. First published November 18, 2004; doi:10.1152/ajpregu.00561.2004.—We reported previously that intravenously administered d-glucose acts in the central nervous system to inhibit gastric motility induced by hypoglycemia in anesthetized rats. The purpose of this study was to determine whether this effect is due to inhibition of dorsal motor nucleus of the vagus (DMV) cholinergic motoneurons, which synapse with postganglionic cholinergic neurons, or to excitation of DMV cholinergic neurons, which synapse with postganglionic nonadrenergic, noncholinergic (NANC) neurons, particularly nitrergic neurons. Three approaches were employed: 1) assessment of the efficacy of d-glucose-induced inhibition of gastric motility in hypoglycemic rats with and without inhibition of nitric oxide synthase [10 mg/kg iv nitro-L-arginine methyl ester (L-NAME)], 2) glycemic rats with and without inhibition of nitric oxide synthase [10 mg/kg iv nitro-L-arginine methyl ester (L-NAME)], and 3) determination of c-Fos expression in DMV neurons after intravenous d-glucose was administered to normoglycemic rats. Results obtained demonstrated a significant contributing component of D-glucose-induced inhibition of hypoglycemia to excite the inhibitory NANC pathway, and the signaling molecule for NANC neurons is thought to be nitric oxide (10, 14, 38, 40).

Data accumulated over the past 15 years suggest that endogenously occurring brain peptides, such as atrial natriuretic factor (18), oxytocin (19), corticotrophin releasing factor (15), and substance P (13), evoke a decrease in gastric motility because they act in the DMV to excite the inhibitory NANC pathway. Furthermore, vagovagal reflex activation, which inhibits gastric motility such as esophageal and antral distension, is also thought to engage the inhibitory NANC pathway (10, 28, 30). Recently, the inhibitory NANC pathway has also been implicated in mediating gastric motility changes associated with alterations in circulating glucose levels. Zhou et al. (47) suggested that glucose “facilitates” the vagal cholinergic pathway that synapses with the stomach nitric oxide pathway to cause gastric relaxation. Moreover, Yuan and Yang (45) described results showing that insulin-induced hypoglycemia causes vagally mediated changes in activity of nitric oxide neurons in the gastric plexuses.

The overall purpose of the present study was to increase our knowledge of how glucose alters vagal outflow to the stomach and decreases gastric motility. Specifically, we sought to determine whether intravenous glucose inhibits gastric motility, in part, or entirely, by activating a NANC inhibitory vagal pathway.

METHODS

Animals and Anesthesia

Adult male Sprague-Dawley rats weighing 300–400 g (Taconic, Germantown, NY) were housed in controlled conditions of room temperature (22°C) and light (12:12-h light-dark cycle) with free access to food and water. Before each experiment, food was withheld overnight, but water was provided ad libitum. Animals were anesthetized with an intraperitoneal injection of a mixture of urethane (800 mg/kg) and α-chloralose (60 mg/kg) dissolved in 0.9% saline. Toe-pinch and corneal reflexes were performed to assess the depth of anesthesia. Glucose does not activate nonadrenergic, noncholinergic inhibitory neurons in the rat stomach

WE RECENTLY REPORTED THAT INTRAVENOUS GLUCOSE INFUSION IN ANESTHETIZED RATS DURING INSULIN-INDUCED HYPOGLYCEMIA PRODUCES A ROBUST AND CONSISTENT INHIBITION OF Gastric motility (34). This inhibitory effect occurred immediately (within 30 s to 2 min) after the start of infusion. Most impressive was the small change in blood glucose level needed to evoke inhibition of gastric motility. In most studies, the change in blood glucose level required to evoke complete inhibition of hypoglycemia-induced increases in antral motility ranged between 3 and 5 mg/dl.

In the above-cited study (34) and in our laboratory’s earlier study (4), we concluded that intravenous glucose inhibited gastric motility via effects in the central nervous system (CNS) rather than in the periphery by modulating vagal outflow to the stomach. This conclusion fits with data indicating that the opposite state, namely hypoglycemia, increases gastric motility by a CNS action leading to increased vagal effects on the stomach (2, 35).

Data accumulated over the past 15 years suggest that endogenously occurring brain peptides, such as atrial natriuretic factor (18), oxytocin (19), corticotrophin releasing factor (15), and substance P (13), evoke a decrease in gastric motility because they act in the DMV to excite the inhibitory NANC pathway. Furthermore, vagovagal reflex activation, which inhibits gastric motility such as esophageal and antral distension, is also thought to engage the inhibitory NANC pathway (10, 28, 30). Recently, the inhibitory NANC pathway has also been implicated in mediating gastric motility changes associated with alterations in circulating glucose levels. Zhou et al. (47) suggested that glucose “facilitates” the vagal cholinergic pathway that synapses with the stomach nitric oxide pathway to cause gastric relaxation. Moreover, Yuan and Yang (45) described results showing that insulin-induced hypoglycemia causes vagally mediated changes in activity of nitric oxide neurons in the gastric plexuses.

The overall purpose of the present study was to increase our knowledge of how glucose alters vagal outflow to the stomach and decreases gastric motility. Specifically, we sought to determine whether intravenous glucose inhibits gastric motility, in part, or entirely, by activating a NANC inhibitory vagal pathway.

METHODS

Animals and Anesthesia

Adult male Sprague-Dawley rats weighing 300–400 g (Taconic, Germantown, NY) were housed in controlled conditions of room temperature (22°C) and light (12:12-h light-dark cycle) with free access to food and water. Before each experiment, food was withheld overnight, but water was provided ad libitum. Animals were anesthetized with an intraperitoneal injection of a mixture of urethane (800 mg/kg) and α-chloralose (60 mg/kg) dissolved in 0.9% saline. Toe-pinch and corneal reflexes were performed to assess the depth of...
anesthesia. Body temperature was monitored by a rectal thermometer and maintained at 37 ± 1°C with an infrared heating lamp. At the end of each experiment, all rats were killed with an overdose of pentobarbital sodium. Research described in this paper fully conforms to National Institutes of Health guidelines and were approved by the Georgetown University Animal Care and Use Committee.

General Procedures

Rats were intubated to maintain an open airway and so that artificial respiration could be instituted when necessary. The carotid artery and jugular vein were cannulated with polyethylene tubing (PE-50) to monitor blood pressure and to administer intravenous drug and glucose infusions, respectively. Blood pressure was recorded by using a bridge amplifier connected to a Power Lab (ADInstruments, Mountain View, CA) data-acquisition system. An incision lateral to the midline was made, exposing the stomach and enabling removal of excess stomach contents through a small incision in the fundus. Tonic and phasic gastric contractions were measured with a latex balloon inserted into the stomach via the fundus and attached to a pressure transducer with polyethylene tubing (PE-160). The balloon was filled with warm deionized water (4–5 ml) to achieve a basal tone of 4–10 mmHg. The pressure transducers were connected to a bridge amplifier coupled to the Power Lab data-acquisition system (ADInstruments). Data were stored on a computer for analysis at a later time.

Experimental Protocols

The effect of D-glucose administered intravenously was evaluated on gastric motility occurring under the following experimental conditions: 1) nitro-L-arginine methyl ester (L-NAME) inhibition of nitricergic transmission and 2) bethanechol-induced gastric motility. The protocols used for each of these experimental conditions are described below. Glucose infusion was performed by using a standard protocol for all studies, which was as follows: a 25% d-glucose solution was infused at a rate of 2 ml/h iv by a syringe pump (Razel Scientific Instruments, Stamford, CT). D-Glucose (25%) was chosen because it results in rapid elevation of blood glucose but is not so viscous that resistance to its infusion occurs. The rate of infusion was kept low to avoid excessive volume expansion. Glucose levels were obtained by analysis of blood samples taken from the tip of the tail. Analysis was carried out by using a Freestyle glucometer (Therasense, Alameda, CA).

L-NAME effect on glucose inhibition to insulin-induced activity. Once a 10-min duration of stable baseline of gastric motor activity was obtained with intragastric balloon recordings, insulin (2.5 IU/animal) was administered subcutaneously to induce hypoglycemia. This insulin dose was based on the results from our previous study (34). Once the hypoglycemia-induced gastric hyperactivity was established, L-NAME (10 mg/kg) was given intravenously. After ~10 min, the responses to L-NAME (i.e., increase of blood pressure and gastric pressure) had stabilized, and then 25% D-glucose solution was infused until the inhibition of gastric motility occurred. Blood glucose levels were obtained before insulin was administered, at the time that robust increases in gastric motor activity due to hypoglycemia were noted, at the time of the L-NAME effect, during the 1- to 6-min period immediately after the glucose infusion was started, and during the recovery period after glucose infusion was terminated. Bethanechol effect on glucose inhibition of insulin-induced activity. For this study, we followed the above general surgical procedure; in addition, the femoral vein in the animals was cannulated with polyethylene tubing (PE-50) for bethanechol-continuous infusion. After stable baseline was obtained and after insulin (2.5 IU/animal)-induced gastric hyperactivity was present, 25% D-glucose infusion (2 ml/h) was started. Once the glucose inhibitory effect was clearly established, bethanechol (30 μg·kg⁻¹·min⁻¹) was infused while the 25% D-glucose infusion continued. Blood samples were taken before insulin was administered, at the time that robust increases in gastric motor activity were noted, during the 1- to 6-min period immediately after the glucose infusion was started, and at the peak bethanechol effect, which was right before the termination of glucose and bethanechol infusion. Control experiments were performed with saline and l-glucose. Instead of giving insulin subcutaneously, animals were given the same volume (0.5 ml) of saline injection, and then a waiting period occurred equal to the time required for the insulin effect; this was then followed by a 25% l-glucose infusion at the same speed (2 ml/h) as 25% D-glucose.

c-Fos immunohistochemistry. Male Sprague-Dawley rats (300–400 g) were allowed to acclimate to the facility for at least 5–7 days with free access to regular chow and water. Five days before the study, animals were given injection of Fluoro-Gold (0.8 mg ip). In the glucose infusion group, we inserted jugular venous catheters into the right jugular vein using methods described previously (26). Animals were acclimated to experimental procedures (i.e., infusion and intravenous or subcutaneous injection) for 3 days before the study. All studies were performed in conscious animals. Food was withheld (for glucose infusion) or given in a limited amount (for insulin injection) the night before experiments. Water was provided ad libitum, but animals were denied access from 8:00 AM on the day of study. In the glucose infusion group, 25% D-glucose or saline was infused at 2 ml/h for 60 min. In the CCK-treated group, CCK-8 (10 and 50 μg/kg) was administered intravenously. In the insulin group, 2.5 IU insulin or an equal volume of saline was administered subcutaneously, and then animals were allowed 60 min to develop hypoglycemia. Sixty minutes after the termination of glucose infusion, CCK injection, or establishment of hypoglycemia, animals were anesthetized with an overdose of pentobarbital sodium (80 mg/kg). This waiting time was chosen on the basis of previous studies, which found that c-Fos immunoreactivity in hypothalamic and brain stem neurons peaks 60–90 min after stimulation (25, 43). After animals showed no response to toe pinch, the thoracic cavity was opened, the inferior vena cava was clamped, and an 18-gauge over-needle Teflon catheter was inserted into the apex of the heart and routed to the entrance of the aorta. Five hundred units of heparin were injected into the catheter, and the right atrium was punctured to allow drainage. The animal was then perfused transcardially with 200 ml of 0.15 M NaCl containing 2% sodium nitrite followed by 200 ml of phosphate-buffered 4% paraformaldehyde containing 2% acrolein followed by another 200 ml of 0.15 M NaCl containing 2% sodium nitrate. The brains were harvested, postfixed for 15 min. In the CCK-treated group, CCK-8 (10 and 50 μg/kg) was administered intravenously. In the insulin group, 2.5 IU insulin or an equal volume of saline was administered subcutaneously, and then animals were allowed 60 min to develop hypoglycemia. Sixty minutes after the termination of glucose infusion, CCK injection, or establishment of hypoglycemia, animals were anesthetized with an overdose of pentobarbital sodium (80 mg/kg). This waiting time was chosen on the basis of previous studies, which found that c-Fos immunoreactivity in hypothalamic and brain stem neurons peaks 60–90 min after stimulation (25, 43). After animals showed no response to toe pinch, the thoracic cavity was opened, the inferior vena cava was clamped, and an 18-gauge over-needle Teflon catheter was inserted into the apex of the heart and routed to the entrance of the aorta. Five hundred units of heparin were injected into the catheter, and the right atrium was punctured to allow drainage. The animal was then perfused transcardially with 200 ml of 0.15 M NaCl containing 2% sodium nitrite followed by 200 ml of phosphate-buffered 4% paraformaldehyde containing 2% acrolein followed by another 200 ml of 0.15 M NaCl containing 2% sodium nitrate. The brains were harvested, postfixed overnight in phosphate-buffered 4% paraformaldehyde, and then stored in 25% sucrose until sectioned. Brain stems were cut into sequential 25-μm coronal sections using a freezing-stage microtome (Jung Histostide 2000, Deerfield, IL). The sections were collected in serially ordered sets through the rostrocaudal extent of the DMV so that each set contained a 1:6 series of hindbrain sections spaced ~150 μm apart. The sections were stored at ~20°C in tissue culture dishes containing cryoprotectant (44) until they were processed.

To ensure that the immunohistochemical analyses were representative of the entire extent of the sectioned brain area, each analysis consisted of sections that were cut ~150 μm apart (every 6th section). The tissue was rinsed with PBS and treated with a solution of 1% sodium borohydride for 20 min. Next, the tissue was incubated for 48–72 h at 4°C with a rabbit-derived antibody directed against the amino terminal of c-Fos (diluted 1:100,000 in PBS containing 0.4% Triton X-100). The tissue was then incubated for 1 h at room temperature with a biotinylated goat anti-rabbit IgG (diluted 1:600 in PBS-Triton X-100). Finally, the tissue was incubated for 1 h at room temperature with avidin and biotinylated horseradish peroxidase (Vectorstain Elite ABC kit, 4.5 μl of reagents A and B per milliliter, in PBS-0.4%-Triton X-100). The presence of the antibody-peroxidase complex was detected by incubating with 25 mg/ml nickel sulfate, 0.2 mg/ml 3,3′-diaminobenzidine, and hydrogen peroxide (0.8 μl of 30% H2O2 per milliliter) in 0.175 M sodium acetate for 10–20 min. This reaction product was black. To identify Fluoro-Gold-containing neurons, the same sections were double stained with an antibody directed against Fluoro-Gold, diluted 1:70,000 in PBS-Triton X-100. Peroxidase was attached to the antibody as described above, and the
presence of the peroxidase was detected by incubating with 3,3'-
diaminobenzidine and hydrogen peroxide in 0.05 M Tris-buffered
NaCl (pH 7.2; 0.15 M). This reaction product was light brown. Through-
out the staining procedure, the tissue was rinsed in PBS multiple times
after each incubation step. The tissue was mounted on Superfrost Plus
glass slides (Fisher Scientific), air-dried overnight, serially dehydrated in
alcohol, cleared in Histoclear, and coverslipped with Histomount.

Tissue slices were visualized with a Nikon Eclipse E600 micro-
scope fitted with a linear encoder (type MSA 001-6, RSF Electronics,
Rancho Cordova, CA) connected to a digital readout device (Micro-
scope fitted with a linear encoder (type MSA 001–6, RSF Electronics,
Rancho Cordova, CA) connected to a digital readout device (Micro-
code II, Boeckeler Instruments, Tucson, AZ), a video camera (DEI-
750, Optronics Engineering, Goleta, CA), and a microcomputer run-
ning the Bioquant software package (R&M Biometrics, Nashville,
TN). The tissue slices were visualized by $\times 10$ and $\times 20$ objective
lenses, and the brain regions of interest [medial subnucleus of the
tractus solitarius (mNTS) and DMV] were outlined by using Paxinos
and Watson (24) as a guide. The numbers of total c-Fos-positive cells
in the mNTS and the numbers of c-Fos-positive and Fluoro-Gold-
positive immunoreactive cells in the DMV were counted separately
on each section. Using the Bioquant software package, we marked each
individual immunoreactive cell during the counting process, eliminat-
ing the possibility of double-counting identified cells. For each ani-
mal, single- or double-labeled neurons in each section were summed
from all sections that were usually 1,050 µm rostral to the calamus
scriptorius to 1,500 µm caudal to the calamus scriptorius. The total
number of positive cells in the area counted was then divided by the
number of animals, and the results were expressed as c-Fos-positive
(mNTS) or c-Fos-positive plus Fluoro-Gold-positive (DMV) neurons
per section. The DMV area was defined by drawing dotted lines along
the Fluoro-Gold-labeled cells. Although only clustered cells were
included, isolated outliers were excluded. c-Fos-positive cells within
the defining area were counted as “c-Fos-positive in DMV.” Occa-
sionally, there were c-Fos-positive cells located right across the lines;
only those located below the DMV area but above central canal were
counted as c-Fos-positive in DMV; otherwise, they were counted as
“c-Fos-positive in NTS.”

Analysis of Gastric Motor Activity and Statistical Analysis

Sum and frequency of gastric contractions were analyzed over a
5-min period. Only intragastric balloon pressure changes of ≥1.0
mmHg were considered as significant contractions and were analyzed.
Means and standard errors of the means were calculated with Sig-
maStat. Data were analyzed with either Student’s paired t-test or
one-way ANOVA (in the case of multiple comparisons) followed by
the Student-Newman-Keuls post hoc test. Differences were consid-
ered statistically different when $P < 0.05$.

Drugs and Source

Insulin (Humulin R) was purchased from Eli Lilly (Indianapolis,
IN). Fluoro-Gold was purchased from Fluorochrome (Denver, CO).
Acrolein was purchased from Polysciences (Warrington, PA). c-Fos
antibody was purchased from Oncogene Sciences (Manhasset, NY).
Biotinylated goat anti-rabbit IgG and Vectastain Elite ABC kit were
purchased from Vector Laboratories (Burlingame, CA). Fluoro-Gold
antibody was purchased from Chemicon (Temecula, CA). Histoclear
and histomount were purchased from National Diagnostics (Atlanta,
GA). All other drugs were purchased from Sigma (St. Louis, MO). All
drugs were dissolved in 0.9% saline. The pH of intravenous drug
solutions was between 7.0 and 7.4.

RESULTS

Effect of Pretreatment with L-NAME on Glucose-Induced
Inhibition of Insulin-Evoked Gastric Contractions

In three rats not pretreated with L-NAME (i.e., control
animals), insulin administered subcutaneously increased gas-
tric contractions, as reflected by an increase in total contraction
amplitudes over 5 min and frequency of contractions (Table 1, Fig. 1A). These increases occurred on average 75.7 ± 7.3 min
after insulin injection and were associated with a decrease of
blood glucose from 87 ± 9 to 25 ± 5 mg/dl. Baseline
intragastric pressures were not significantly altered by insulin
(Table 1). At the time of the increases in gastric contractions,
D-glucose (25% D-glucose at 2 ml/h iv) was administered and
inhibited contractions within 3.3 ± 0.9 min (Fig. 1A). Blood
glucose levels measured at the time of glucose-induced inhib-
iton of gastric contractions averaged 36 ± 13 mg/dl.

In five additional rats, L-NAME was administered on aver-
age at 76.4 ± 5.6 min after subcutaneously administered
insulin. At this time, insulin increased the total contraction
amplitudes over 5 min from 0 to 66 ± 11 mmHg (Table 1, Fig.
1B). Contraction frequency at this time was 4.8 ± 0.6 contrac-
tions/min. No significant change was observed in intragastric
pressure. L-NAME administered intravenously at a dose of 10
mg/kg produced an immediate increase in gastric contractions
(Table 1, Fig. 1B). The sum of contraction amplitudes over 5
min increased to 127 ± 8 mmHg, and this increase was a
significant change ($P < 0.05$) relative to the value obtained
after insulin alone. Frequency of contractions attained a value
of 6.0 ± 0.3 contraction/min, but this change was not statisti-
cally significant. Intragastric pressure increased after L-
NAME and rose from 7.3 ± 0.6 to 8.4 ± 0.5 mmHg but was not
statistically significant. Blood glucose levels averaged 89 ± 10
mg/dl before insulin and decreased to 23 ± 5 mg/dl after
insulin administration. Addition of L-NAME did not further
alter the blood glucose level (27 ± 5 mg/dl). On average,
10.5 ± 0.8 min after L-NAME was administered, D-glucose
infusion was started and inhibited contractions within 4.1 ±
0.7 min (Table 1, Fig. 1B). Blood glucose levels measured at
the time of glucose-induced inhibition of gastric contractions
averaged 35 ± 5 mg/dl. Most important, the ability of D-
-glucose to inhibit insulin-evoked increases in gastric contrac-
tions was not significantly altered by pretreatment with L-
NAME with regard to either the latency, 3.3 ± 0.9 min for the
test group vs. 4.1 ± 0.7 min for the L-NAME-treated group,
to inhibit gastric contractions or the magnitude of the inhibition
(at the 6- to 11-min time period after glucose contractions were
abolished in both groups) (Table 1).

Effect of D-Glucose-Induced Inhibition of Gastric
Contractions on Bethanechol-Evoked Gastric Responses

Our rationale for these experiments was based on the idea
that, if glucose acts to stimulate a vagal NANC inhibitory
pathway to the stomach, gastric contractions evoked by
bethanechol would be significantly reduced. Alternatively, if
pharmacologic action would not be diminished.

The test protocol in these studies was first to administer
insulin subcutaneously to increase gastric contractions. Next,
D-glucose was infused intravenously to inhibit gastric contrac-
tions. Finally, with D-glucose infusion continuing, bethanechol
was administered at 30 µg·kg⁻¹·min⁻¹, and peak increases in
gastric contractions were noted. The data obtained from five
experiments are summarized in Table 2, and a representative
Table 1. Effects of L-NAME on D-glucose-induced inhibition of gastric motility

<table>
<thead>
<tr>
<th>Treatment and Groups</th>
<th>Effect 1–6 min</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin and L-NAME</td>
<td>F, min⁻¹</td>
<td>F, min⁻¹</td>
</tr>
<tr>
<td>Insulin</td>
<td>7.0 ± 1.8</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>L-NAME</td>
<td>7.0 ± 1.8</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>6.0 ± 0.5</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>D-Glucose + L-NAME</td>
<td>6.0 ± 0.5</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE. AMP, sum of amplitudes; F, frequency of phasic contractions; IGP, intragastric pressure.

Fig. 1. Representative chart recordings from two experiments in which 25% D-glucose infusion (2 ml/h) inhibited insulin-induced gastric contractions in an animal of the control group (A) and an animal given nitro-L-arginine methyl ester (L-NAME) after subcutaneously administered insulin (B). A: blood glucose (bgl) was 71 mg/dl before insulin administration. Next, at a recording break, 2.5 IU sc insulin was administered; 68 min later, the frequency of contractions had increased. That is, on average, at 19.0 ± 4.4 min after the start of bethanechol infusion, both the sum of contraction amplitudes over 5 min and the frequency of contractions were significantly increased from 18 ± 8 mmHg and 1.7 ± 0.7 contractions/min to 112 ± 20 mmHg and 5.7 ± 0.5 contractions/min, respectively (Table 2).

The protocol for the control experiments (n = 4) was to administer insulin vehicle (physiological saline) subcutaneously, wait 66.3 ± 3.1 min, and then administer 25% D-glucose at 2 ml/h in place of D-glucose [L-glucose at this dose exhibits no effect on gastric motor activity (34)]. With L-glucose infusion continuing, bethanechol infusion (30 μg·kg⁻¹·min⁻¹) was started as described above. The increases in gastric motility observed at 19.0 ± 2.0 min after the start of bethanechol infusion were as follows: sum of contraction amplitudes over 5 min increased from 10 ± 2 to 70 ± 12 mmHg (P < 0.05), and the frequency of contractions increased from 0.8 ± 0.3 to 4.2 ± 1.2 contractions/min (P < 0.05). Thus, in the presence of L-NAME, the inhibition of gastric motility caused by D-glucose infusion was significantly different compared with baseline values using one-way ANOVA, followed by Student-Newman-Keuls post hoc test.
Table 2. Effects of D-glucose on bethanechol-induced gastric motility

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>Baseline</th>
<th>Insulin/Saline Effect</th>
<th>Glucose Effect</th>
<th>Bethanechol Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>∑ AMP, mmHg</td>
<td>IGP, mmHg</td>
<td>F, min⁻¹</td>
<td>∑ AMP, mmHg</td>
</tr>
<tr>
<td>Saline and l-glucose (n = 4)</td>
<td>26±14</td>
<td>5.2±1.8</td>
<td>13±4</td>
<td>6.8±2.4</td>
</tr>
<tr>
<td>Insulin and D-glucose (n = 5)</td>
<td>10±4</td>
<td>1.0±0.4</td>
<td>8.4±0.8</td>
<td>4.8±0.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Statistically significant difference compared with baseline values using one-way ANOVA, followed by Student-Newman-Keuls post hoc test. †Statistically significant difference compared with glucose effect values using one-way ANOVA, followed by Student-Newman-Keuls post hoc test.

of D-glucose-induced inhibition of gastric contractions, the excitatory effect of bethanechol on gastric motor activity was not attenuated. Indeed, the excitatory effect of this muscarinic receptor agonist actually appeared to be somewhat enhanced compared with corresponding data obtained from the control experiments.

Effect of D-Glucose Infusion on c-Fos Expression in DMV Neurons Projecting to the Stomach

As mentioned in METHODS, these studies were performed in conscious rats. In our first study, insulin was administered subcutaneously and 60 min later, 25% D-glucose infusion was begun and continued for 3–5 min. This protocol was similar to the protocol used for the control studies carried out in anesthetized rats (see protocol for studies carried out in Table 1). One hour after D-glucose infusion was terminated, brains were harvested, and the DMV area was examined for c-Fos expression. Three experiments of this type were performed, and five control experiments were conducted where saline vehicle (instead of D-glucose) was infused for 3–5 min after subcutaneous insulin administration. Data obtained indicated that insulin increases c-Fos in the DMV to the same extent in both n-glucose-infused animals and the saline-infused animals. Assuming that insulin excites the vagal-cholinergic excitatory path to the stomach, we hypothesized that, if D-glucose activates the vagal-NANC pathway, then more c-Fos expression would be observed in the insulin plus D-glucose group compared with the control group. However, this did not occur: in five control rats treated with insulin followed by isotonic saline administration, the total number of cells expressing c-Fos in the dorsal vagal complex (NTS + DMV) was 2,084 ± 60, whereas in three rats treated with the same dose of insulin followed by D-glucose infusion, 1,953 ± 77 cells were activated to express c-Fos. It is possible that some activation of the vagal-NANC pathway did occur but was dwarfed by a more powerful excitatory effect of insulin on the vagal-cholinergic pathway.
Table 3. Effect of \(\alpha\)-glucose, CCK, and hypoglycemia on c-Fos expression in NTS and DMV neurons and on c-Fos expression in DMV neurons projecting to the stomach

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>c-Fos+ in NTS and DMV</th>
<th>c-Fos+, FG+ in DMV, % of total c-Fos</th>
<th>FG+ in DMV</th>
<th>c-Fos+, FG+ in DMV, % of total FG</th>
</tr>
</thead>
<tbody>
<tr>
<td>15-min Intravenous infusion</td>
<td>114 ± 32</td>
<td>3 ± 1 (2.6 ± 0.6)</td>
<td>1351 ± 49</td>
<td>2 ± 2 (0.2 ± 0.2)</td>
</tr>
<tr>
<td>Saline ((n = 4))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose ((n = 3))</td>
<td>72 ± 37</td>
<td>2 ± 1 (2.5 ± 1.2)</td>
<td>1377 ± 92</td>
<td>0</td>
</tr>
<tr>
<td>Intravenous bolus injection</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline ((n = 3))</td>
<td>19 ± 9</td>
<td>0</td>
<td>889 ± 162</td>
<td>†</td>
</tr>
<tr>
<td>CCK-8 ((n = 6))</td>
<td>1.455 ± 218*</td>
<td>166 ± 29* (11.5 ± 0.9)*</td>
<td>846 ± 120</td>
<td>1 ± 1 (0.2 ± 0.1)</td>
</tr>
<tr>
<td>Subcutaneous administration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline (hypoglycemia control) ((n = 2))</td>
<td>237 ± 184</td>
<td>12 ± 9 (5.4 ± 0.4)</td>
<td>1.563 ± 55</td>
<td>20 ± 19 (1.4 ± 1.3)</td>
</tr>
<tr>
<td>Insulin (hypoglycemia) ((n = 6))</td>
<td>1.813 ± 120*</td>
<td>118 ± 18* (6.3 ± 0.7)†</td>
<td>1.324 ± 29*‡</td>
<td>283 ± 14† (21.5 ± 1.3)‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. DMV, dorsal motor nucleus of the vagus; FG, Fluoro-Gold; NTS, nucleus tractus solitarius; +, positive. *Statistically significant difference compared with its own saline control values using \(t\)-test. †Statistically significant difference compared with corresponding control values of the other treatment groups using one-way ANOVA followed by Student-Newman-Keul post hoc test. ‡Statistically significant different compared with corresponding value of the CCK-8 IV bolus injection treatment group using \(t\)-test.

pathway. Hence, we next tried \(\alpha\)-glucose infusion without insulin. Seven animals were given 25% \(\alpha\)-glucose (\(n = 3\)) or saline (\(n = 4\)) infusion for 15 min. Sixty minutes after the termination of infusion, brains were harvested and examined for c-Fos and c-Fos plus Fluoro-Gold expression (Fluoro-Gold was administered intraperitoneally 5 days earlier to label DMV-projecting neurons as described in METHODS). Data are summarized in Table 3 and indicate that 15 min of 25% \(\alpha\)-glucose infusion did not increase c-Fos expression in either the combined areas of the NTS and DMV or the DMV alone above that observed in control animals, i.e., in animals that received 15 min of saline infusion. Likewise, \(\alpha\)-glucose infusion did not increase c-Fos expression in stomach-projecting DMV neurons as defined by Fluoro-Gold staining.

Another inhibitor of rhythmic gastric motility, CCK-8 (1), was also tested for evidence of stimulation of a vagal-NANC pathway. Two doses of CCK-8 (\(n = 6\)) and saline vehicle (\(n = 3\)) were injected as an intravenous bolus, and brains were harvested 60 min after intravenous injection. Both doses of CCK-8 (i.e., doses of 10 and 50 \(\mu\)g) produced similar effects; therefore, these data were combined. The data showed that CCK-8 activated significant amounts of c-Fos in the NTS and DMV combined area and in the DMV alone compared with the intravenous saline control group (Table 3). Most important, although significant c-Fos expression was detected within the DMV area after CCK-8, the c-Fos-positive cells were not DMV neurons that projected to the stomach as defined by Fluoro-Gold retrograde labeling (Table 3 and Fig. 3). Indeed, 11.5 ± 0.9% of the c-Fos expressing neurons in the NTS ± DMV area were located within the boundaries of the DMV, but only 0.2 ± 0.1% were in Fluoro-Gold-stained neurons. Finally, there were fewer DMV neurons labeled by Fluoro-Gold compared with the saline controls of the other two treatment groups (Table 3).

To confirm the ability of DMV neurons that project to the stomach to express c-Fos, animals that had already received Fluoro-Gold injection were given 2.5 IU insulin (\(n = 6\)) or the same volume of saline vehicle (\(n = 2\)) subcutaneously. After a period of 60 min for animals to develop hypoglycemia, another 60 min was allowed before brains were harvested for immunohistochemistry processing. Data obtained are presented in Table 3 and Figs. 3B and 4. As shown in Table 3, insulin-induced hypoglycemia increased c-Fos expression in neurons in the NTS and DMV combined area and in the DMV alone compared with saline controls. The double-labeled neurons occurred mainly in the medial portion of the nucleus. This was true of all animals studied. Figure 4 shows the rostral-caudal pattern of c-Fos expression present in the DMV along with the rostral-caudal pattern of double-labeled neurons. As can be
The purpose of this study was to determine whether intravenously administered glucose inhibits gastric motility, in part, or entirely, by activating an inhibitory NANC vagal pathway, as has been suggested by a previous study (47). Three experimental approaches were taken. The first was based on the assumption that the NANC inhibitory output pathway includes a significant component of nitrergic neurons. This assumption was derived from findings demonstrating that drugs that inhibit nitric oxide synthase (NOS) counteract vagal nerve-induced inhibition of gastric motility (10, 14, 38, 40). In this case, prior treatment with a NOS inhibitor such as L-NAME should inhibit or decrease the response to intravenously administered glucose if this were in fact mediated by NANC (nitrergic) neurons. The second approach was based on assessing gastric contractions evoked by the peripherally acting muscarinic receptor agonist bethanechol. We reasoned that if glucose acts to stimulate a vagal NANC inhibitory pathway to the stomach, gastric contractions evoked by bethanechol would be significantly reduced regardless of the neurotransmitters comprising the NANC neuron. On the other hand, if glucose acts primarily via the other parallel pathway, i.e., inhibition of vagal cholinergic excitatory output, gastric contractions evoked by a peripherally acting agent such as bethanechol would not be diminished. The third approach was based on the assumption that if glucose acts to stimulate vagal NANC inhibitory output, then c-Fos expression should be evident in a subset of stomach-projecting DMV neurons after intravenous glucose administration.

Our findings were as follows: 1) prior treatment with L-NAME in a dose that others have shown will block NANC influence on gastric motility, i.e., 10 mg/kg iv (10, 38), had no effect on glucose-induced inhibition of gastric motility; 2) bethanechol-induced increases in gastric motility were not attenuated by the presence of gastric motility-inhibiting doses of glucose (and, indeed, bethanechol-induced increases in gastric motility were enhanced); and 3) no significantly appreciable c-Fos expression in stomach-projecting DMV neurons occurred after glucose dosing. These results lead us to conclude that glucose does not activate NANC neurons in the rat stomach. By exclusion, we suggest that glucose inhibits hypoglycemia-induced gastric motility primarily by acting in the CNS and inhibiting the excitatory path consisting of vagal preganglionic neurons synapsing onto cholinergic myenteric neurons.

Consistent with our findings are several additional findings. First, studies of the effects of glucose on spontaneously occurring efferent vagal preganglionic nerve activity revealed that glucose injected into the carotid artery of anesthetized rats immediately suppressed the efferent activity in the gastric vagus nerve (8, 33). Second, studies of neural activity in the DMV of rats indicated that intravenously administered glucose infusion significantly reduces this activity (37). In that study, extracellular single unit activity was recorded from the DMV area, and glucose infusion reduced neural firing from 16.2 ± 2.6 to 5.0 ± 1.4 discharges/s. Third, findings of the present study demonstrated that CCK-8, a neuropeptide well known to inhibit rhythmic gastric motility in part by reflexively influencing parasympathetic outflow to the stomach (39), also did not increase c-Fos expression in stomach-projecting DMV neurons. This latter finding agrees with the earlier report of Rinaman et al. (25), who found relatively little c-Fos expression in the DMV of rats after CCK-8 administration. Furthermore, Takahashi and Owyang (39) found that prior treatment of rats with the NOS inhibitor L-NAME had no effect on CCK-8-induced inhibition of gastric motility, consistent with a lack of engagement of NANC neurons with this inhibitor of rhythmic gastric motility as well.

The lack of a role for the NANC pathway in the stomach-relaxing effect of glucose and CCK-8 is also in agreement with our data obtained with nicotine administration (5) and esophageal distension (31). In these studies, both intravenously administered nicotine and esophageal distension in the rat cause gastric relaxation that appears to be mediated by vagal efferent neurons. However, analogous studies carried out following intravenous nicotine administration and esophageal distension similarly revealed a lack of significant expression of c-Fos in stomach-projecting DMV neurons, suggesting predominantly decreased rather than increased vagal activity.

Our inability to show a positive role for activation of NANC neurons in the rat stomach using experimental interventions that inhibit gastric motility, such as intravenous glucose and CCK-8 in hypoglycemic rats, intravenous nicotine, and esophageal distension, compels us to examine the strength of evidence from previous studies supporting a role of this pathway. The NANC pathway has been implicated in the gastric relaxation produced by centrally administered oxytocin (19). However, Verbalis and colleagues (22) indicated that oxytocin, like
intravenous glucose and CCK-8, does not increase c-Fos expression in the DMV. Atrial natriuretic factor, substance P, and corticotrophin-releasing hormone have all been described as activating the vagal preganglionic neurons synapsing onto NANC myenteric neurons (12, 15, 18). Evidence for this conclusion was based on experiments showing that gastric relaxation caused by locally applied drug to the DMV could be prevented by prior bilateral cervical vagotomy. Anatomically, the DMV is in close proximity to the medial subnucleus of the tractus solitarius (mNTS), making it difficult to be sure that a gastric response evoked by microinjecting a drug into the DMV originates solely from the DMV. In fact, bilateral cervical vagotomy will also prevent gastric relaxation evoked from local drug application to mNTS (6). A more definitive test of a direct DMV effect is the demonstration that ipsilateral vagotomy will prevent an inhibitory effect on gastric motility, but ipsilateral vagotomy was not performed in these studies. [Note: ipsilateral vagotomy does not appear to block gastric motility responses evoked from the mNTS (6).] Evidence that esophageal distension induced gastric relaxation-invoked activation of the NANC pathway was based, in part, on the finding that esophageal distension excites DMV neurons that comprise fibers of the efferent cervical vagus nerve (28). The difficulty in interpreting these data is that it is not clear whether the nerves identified in this study actually innervate the stomach. Finally, Ishiguchi et al. (10) concluded that distension of the antrum results in reflex activation of a NANC pathway. Their evidence was based on data showing that intravenous L-NAME blocked reflex-induced inhibition of the pylorus. Unfortunately, in this study, reflex function was tested 15 min after L-NAME administration. By this time, L-NAME accumulates in the NTS to such an extent that it reduces spontaneous firing of mNTS neurons (17). Because nitric oxide in the NTS is essential for the function of the vagovagal reflex (5, 31), one cannot conclude from these results that NANC neurons in the rat stomach are involved in the response.

The absence of c-Fos expression in DMV neurons that project to the stomach after intravenous d-glucose infusion as stand-alone evidence for d-glucose not activating the DMV pathway connected NANC neuron is insufficient evidence for drawing a conclusion. The reason for this is that absence of c-Fos expression does not always indicate an absence of increased neural activity because not all brain cells express c-Fos in response to stimulation (22). However, despite this caveat, the L-NAME and betahanechol data nonetheless do provide evidence against glucose-induced activation of an inhibitory vagal pathway. Also, our c-Fos methodology clearly was able to detect excitation of DMV neurons that project to the stomach following insulin-induced hypoglycemia. Hypoglycemia as shown by others (22, 45) activated c-Fos expression in ~22% of DMV neurons that project to the stomach. The DMV neurons activated by hypoglycemia were located in the medial part of the nucleus (Fig. 3). DMV neurons projecting to the antrum are located mainly in the medial part of the nucleus, whereas DMV neurons projecting to the proximal stomach (e.g., fundus) are located primarily in the lateral part of the nucleus (21, 23). These findings correlate nicely with the effect of insulin-induced hypoglycemia on gastric motility. Hypoglycemia in this study, as well as in our previous study (34), increased phasic antral motility but did not affect tonic contractions that are known to be generated from the proximal stomach (7). In our studies, tonic contractions were reflected in the intragastric pressure values, and these were not significantly affected by insulin-induced hyperglycemia. A separation of functional neural pathways to the antrum and fundus was also noted by Ferreira et al. (5), with nicotine microinjected into the mNTS of the rat. A low dose of nicotine microinjected into the mNTS affected gastric tone, whereas a higher dose was required for evoking effects on phasic antral contractions. Nicotine-induced decreases in gastric tone engaged a CNS circuit that used norepinephrine at α2-adrenoceptors of the DMV, whereas the nicotine-induced decreases in phasic contractions engaged a CNS circuit that used GABA at the DMV. It is tempting to speculate that insulin-induced hypoglycemia might activate antral-projecting DMV neurons by a mechanism involving inhibition of GABA. This would not be surprising, since our previous findings indicate that glucose inhibited antral motility probably by release of GABA at the DMV (4).

Although CCK-8 did not appear to activate DMV neurons that project to the stomach, nonetheless, some cells in the DMV were activated as determined by c-Fos expression (Table 3 and Fig. 3). It is known that two populations of small neurons likely exist in the DMV that do not exit the brain via the vagus nerve (20). One population consists of interneurons or neurons that project for only short distances (20). The other population may project to other regions of the brain. There is evidence of efferent projections to the paraventricular nucleus of the hypothalamus (36), the parabrachial nucleus (11), and cerebellum (46). Two of these three projection areas are known to influence gastric motility [paraventricular nucleus (32) and cerebellum (16)] and might be involved in the CNS pathway mediating the effect of CCK-8 on gastric motility. The data obtained with CCK-8 graphically emphasize the importance of using a retrograde tracer like Fluoro-Gold before drawing conclusions about experimental interventions that appear to induce c-Fos expression in DMV neurons, since c-Fos expression may occur in quite substantial numbers of DMV cells that do not actually project to the stomach (Table 3). In this regard, it is particularly striking that intravenously administered glucose did not activate any neurons in the DMV, whether projecting to the stomach or not.

In summary, our goal was to determine whether glucose-induced inhibition of gastric motility in hypoglycemic rats was caused in part or entirely by activation of the DMV pathway connected to NANC neurons. By pretreating animals with the NOS inhibitor L-NAME, by assessing the ability of betahanechol to stimulate gastric motility during glucose exposure, and by examining DMV neurons for c-Fos expression during exposure to glucose, we found no evidence in support of glucose-induced activation of an inhibitory vagal pathway. Instead, our data are most consistent with a predominant effect of glucose to inhibit excitatory vagal preganglionic neurons synapsing onto cholinergic myenteric neurons.

**GRANTS**

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant RO1 DK-57105 (to R. A. Gillis).

**REFERENCES**

1. Adelson DW, Million M, Kanamoto K, Palanca T, and Tache Y. Coordinated gastric and sphincter motility evoked by intravenous CCK-8...


28. Zheng ZH, Dietrichs E, and Walberg F. The mechanisms of glucose effects on gastric motility and tone evoked by 10.220.33.3 on April 18, 2017 http://ajpregu.physiology.org/ Downloaded from