Glutamate Mediates an Excitatory Influence of the Paraventricular Hypothalamic Nucleus on the Dorsal Motor Nucleus of the Vagus

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Zhang, Xueguo, and Ronald Fogel. Glutamate mediates an excitatory influence of the paraventricular hypothalamic nucleus on the dorsal motor nucleus of the vagus. J Neurophysiol 88: 49–63, 2002; 10.1152/jn.00877.2001. Data have shown that the paraventricular nucleus of the hypothalamus (PVN) and the dorsal motor nucleus of the vagus (DMNV) play important roles in the regulation of gastrointestinal function and eating behavior. Anatomical studies have demonstrated direct projections from the PVN to the DMNV and physiological studies showed that the DMNV mediates many of the effects of PVN stimulation and electrical current stimulation of the PVN excites a subset of DMNV neurons. The aim of this study was to characterize the role of glutamate receptors in the excitatory influence of the PVN on gut-related DMNV neurons. Using single-cell recording techniques, we determined the effects of kynurenic acid, 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX), and DL-2-amino-5-phosphono-pentanoic acid (DL-AP5) on the increase in firing rate due to electrical current stimulation of the PVN. In initial experiments, we studied 24 DMNV neurons excited by electrical current stimulation of the PVN. Kynurenic acid, a broad-spectrum glutamate receptor antagonist, prevented the PVN effect in 22 neurons and significantly attenuated the effect in the other cells. Nine of these neurons demonstrated an inhibition in firing rate with PVN stimulation after pretreatment with kynurenic acid. In a separate group of 12 neurons, we determined the effects of CNQX (1.2 nmol) injected into the DMNV. This AMPA receptor antagonist completely blocked the excitatory response to PVN stimulation of six DMNV neurons and significantly attenuated the response of the other six DMNV neurons. The addition of 1.2 nmol DL-AP5, a N-methyl-D-aspartate (NMDA) receptor antagonist, further attenuated the response to PVN stimulation in four of the five DMNV neurons that were still excited after CNQX treatment. The fifth neuron demonstrated PVN-induced inhibition of firing rate after treatment with CNQX and DL-AP5. In a separate group of 11 DMNV neurons excited by electrical stimulation of the PVN, DL-AP5 partially attenuated the excitatory responses of only four DMNV neurons and did not block the excitation of any cells. The mean latency (14 neurons tested) from the PVN to the DMNV was 37.71 ± 2.40 (SE) ms. Monosynaptic action potentials and excitatory postsynaptic potentials were demonstrated in three DMNV neurons by intracellular recording. Our results indicate that glutamate released from PVN neurons projecting to the DMNV excites the gut-related vagal motor neurons by acting predominantly on the AMPA receptor. The NMDA receptor plays only a minor role in the excitatory effect.

INTRODUCTION

Evidence has suggested that, in the CNS, the hypothalamic paraventricular nucleus (PVN) and the dorsal vagal complex (DVC) are two important structures in the regulation of gastrointestinal function and feeding behavior. Anatomical studies have demonstrated the direct connection between the PVN and DVC. Both retrograde and orthograde (Saper et al. 1976; Swanson and Kuypers 1980; Willett et al. 1987) tracing methods have revealed that there is a substantial projection from PVN to the DVC with most descending fibers terminating in the ipsilateral DVC.

Functionally, the PVN, a higher center controlling the activities of the autonomic nervous and endocrine systems in the mammalian brain, exerts profound influences on gastrointestinal function (Flanagan et al. 1992) and glucoregulation in a vagal dependent manner (Tokunaga et al. 1986b). Furthermore, recent advances reveal that the PVN and vagus nerve are involved in the regulation of energy balance and feeding behavior that may lead to obesity or malnutrition (Kirchgeessner and Sclafani 1988).

As we have reported, the PVN can affect activities of 80% of the dorsal motor nucleus of the vagus (DMNV) neurons that project to the gastrointestinal tract. Both excitation and inhibition were observed following electrical stimulation of the PVN, but the excitatory influence was dominant (59 vs. 41%) (Zhang et al. 1999).

As we have stated previously, the major effect of the PVN activation on the DMNV neurons is excitatory. What are the possible neurotransmitters that mediate the excitatory effect? Neurons containing many neurotransmitters such as bombesin (Costello et al. 1991), oxytocin (Olson et al. 1992), vasopressin, somatostatin, L-enkephalin, and M-enkephalin (Sawchenko and Swanson 1982) have been shown projecting to the DVC. The possible roles of these neurotransmitters have been investigated extensively (Dubois-Dauphin et al. 1992; Mo et al. 1992; Tian and Ingram 1997). However, as mentioned by Sawchenko and Swanson, less than one-fourth of the PVN neurons projecting to the DVC contain recognized neurotransmitters. This suggests that the major neurotransmitters from the PVN to the DVC are unknown (Sawchenko and Swanson 1982). We postulate that glutamate may be responsible for DMNV neurons’ excitatory response to PVN activation. Glutamate functions as an excitatory neurotransmitter throughout the CNS. Glutamate-containing neurons have been located in the PVN (Matsumoto et al. 1994). The excitatory response of the ventrolateral medulla neurons to the electrical stimulation of the PVN can be blocked by glutamate receptor...
antagonist, indicating that the glutamate-containing PVN neurons project down to the brain stem. In the present experiments using electrophysiological recording and microinjection of selected glutamate agonists and antagonists, we found that glutamate is the major, if not the only, neurotransmitter responsible for the excitatory response of the DMNV neurons elicited by electrical stimulation of the PVN.

METHODS

Animal surgery

Adult Sprague-Dawley rats (weighing 271–320 g) were anesthetized with pentobarbital sodium (50 mg/kg ip). Paw pinch reflex in response to punch legs with a forceps and heart rate were continuously monitored, and supplemental doses of pentobarbital sodium were administered intraperitoneally to maintain a deep level of surgical anesthesia and muscle relaxation. The surgical procedure was similar to what was previously reported (Zhang et al. 1995) with some modification. Briefly stated, a plastic tube was inserted into the trachea by tracheotomy for artificial ventilation. A midline incision was made to expose the abdominal organs. A pair of Teflon-coated, pure-gold stimulating electrodes (76 μm OD) was placed around the anterior and posterior branches of the subdiaphragmatic vagal nerves, at a level immediately above the celiac and accessory branches of the vagus nerve. A catheter was inserted into the greater gastric curvature as an influx for the stomach. The pylorus was transected, and two tubes were inserted into the openings. One toward the stomach was used as the gastric efflux and the other was used as intestinal influx. Then a tube was placed at a transection of the intestine 10 cm caudal to the ligament of Treitz. The other end of the intestine was closed with silk sutures. The abdomen was then closed, and a small piece of gauze was used as drainage to prevent an accumulation of secretory fluid in the abdomen (Zhang et al. 1995).

After the abdominal surgery, the animal was placed on a stereotaxic frame. A bipolar stimulating electrode was placed into the PVN 2.0 mm caudal to the bregma, 0.5 mm lateral to the midline, and 8.5 mm from the skull surface (Zhang et al. 1999). The vagal area in the dorsal brain stem was exposed by removing the dorsal part of the neck muscle, the atlanto-occipital membrane, and a part of the occipital bone overlying the cerebellum. A beveled glass micropipette (A–M Systems; tip diameter, 0.08–0.1 μm; R = 50–70 MΩ), filled with 2% neurobiotin in 1.0 M KCl, was lowered into the nucleus of solitary tract (NST) and DMNV. To deliver the specific glutamate antagonist and control solution to the recording area, a three-barrel pipette filled with different solutions was placed near the recording electrode. The carriers of the recording electrode and multibarreled pipette were lowered to the brain at 30° to the horizontal plane. The tips of the pipettes met so they will deliver to the same location. The multibarreled pipette was controlled by micrometer heads driven by electrical minimotors. Because the injection was hydraulic with no air between the solution and the plunges, this specially made apparatus allowed us to deliver nanoliter volumes of different solutions (0.1 nl/scale) through each pipette with continuous infusion or bulk injection. The open part of the brain was covered by 3% agar in saline to limit any displacement due to respiration and heart beat.

In seven animals, we placed a multibarreled pipette at the point of electrical stimulation to stimulate the PVN with glutamate. Glutamate acts only on cell body and not the passing fibers. The results of the electrical and chemical stimulation of the PVN will be compared.

Recording neurons of the DVC

After the surgery, extracellular recording and intracellular injection techniques were used to characterize the DVC neurons. Biphasic electrical pulses (0.5-ms duration, 3–10 V, 1 Hz) were delivered to the subdiaphragmatic vagus nerve. The recording electrode was advanced by 2.5-μm steps. All units driven by the vagal stimulation electrode were tested for a response to gastric and intestinal distention created by raising the efflux catheters to 20 cm of water above the animal. The distention was maintained for 60 s (Zhang et al. 1995).

After characterization of the neuronal responses to the gastrointestinal distention, all units were tested for their responses to electrical stimulation of the PVN. The PVN was stimulated with 1.0-ms duration, 0.1- to 0.3-mA electrical pulses at 15 Hz for 60 s. The neurons that were modulated by PVN electrical stimulation were further characterized by infusion of small volumes of the selected agents based on our preliminary study. Usually the multibarreled pipettes (3 barrels) were filled with an array of solutions such as one with a receptor agonist, the second with a receptor antagonist, and the third with a control solution. Following infusion of the selected agents into the recorded area, the neuronal responses to electrical stimulation of the PVN and/or gastrointestinal distention were re-evaluated. All responses to the PVN activation and gastrointestinal distention were recorded and stored in an IBM compatible computer using Axoscope software (Axon Instruments, Foster City, CA) for further statistical analysis.

Several criteria helped to identify the neurons as either DMNV or NST neurons. Retrograde activation by electrical stimulation of the subdiaphragmatic vagal nerve was used to differentiate DMNV neurons and the NST neurons. This activation is defined as having a constant latency, following a high-frequency electrical stimulation of the vagal nerve (75–150 Hz, see Figs. 3C and 5C for samples) and having a positive collision test. The NST neurons were recognized by their anterograde response that includes variable latency, low-frequency following, and multiple responses to a single electrical stimulation. However, anterograde activation might also be seen in a DMNV neuron if it receives direct projections from the vagal afferents. Ten neurons were intracellularly injected and stained using histochemistry to identify the recorded neurons.

To evaluate whether the excitatory response of the DMNV neurons to electrical stimulation of the PVN is direct or indirect, we intracellu- larly recorded and tested the synaptic events of six DMNV neurons when the PVN was stimulated by electrical current. The response was recorded and the latency of the electrical stimulation of the PVN on the DMNV neurons was analyzed.

Data analysis

Statistical analysis was used to test the significance of the change after the start of stimulus. All results are expressed as means ± SE. Peristimulus time histograms (5-s bins) were constructed for the period beginning 30 s before and ending 90 s after initiation of gastrointestinal distention or PVN stimulation (stimuli were maintained for 60 s). This 120-s trace was divided into four 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, while period 3 represented the late response. Period 4 contained the first 30 s after the stimulus was discontinued and therefore indicated any delayed response or change induced by removal of the stimulus. To determine whether a given response is significantly more or less than the baseline, the mean activity during periods 2–3 were compared with the mean activity during the pre- and poststimulus periods (periods 1 and 4, respectively) using Bonferroni’s approach to post hoc comparisons. Each neuron was analyzed for a response separately as stated in our previous publication (Zhang et al. 1999).

Histology

At the end of the experiment, the rats were administered a lethal dose of pentobarbital sodium (100 mg/kg) and perfused through the heart with 500 ml of 0.9% saline. The rinse solution was followed by
500 ml of a fixative solution containing 1% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (4°C, pH 7.4). The brain was removed from the skull and stored in 20% sucrose overnight at 4°C. To verify the locations of the PVN stimulating electrode, the recording electrode, and multibarreled pipette, the brain including the PVN was transected at 50 μm, mounted on slides, and inspected under microscope. Only were those experiments where the stimulating electrode was located in the PVN used for further analysis.

To view the recorded DMNV neurons, intracellular injection and histochemical reaction were made to identify the properties of the recorded neurons. Briefly, the brain stem were sectioned at 50 μm, incubated in an avidin-horseradish peroxidase solution, and stained in a solution containing diaminobenzidine, cobalt chloride, nickel, and hydrogen peroxide. The neuron was reconstructed using an IBM-compatible computer. The procedure allowed us to document the morphology of the neuron and to identify the NST or DMNV neurons (please see our previous publication for details and Fig. 8A for a sample) (Zhang et al. 1995).

**Solutions**

Three classes of ionotropic glutamate receptors have been identified: N-methyl-D-aspartate (NMDA), AMPA, and kainate receptors (Dingledine and McBain 1994). Both NMDA and AMPA receptors have been identified in the vagal complex and may mediate the PVN excitation of DMNV neurons (Broussard et al. 1997; Lacassagne and Kessler 2000). At present, the study of kainate receptors in synaptic transmission is limited because of the lack of specific receptor antagonists. In the present experiment, we used 10–100 mM kynurenic acid (Sigma–Aldrich, St. Louis, MO) as a glutamate receptor antagonist dissolved in 0.1 M phosphate buffer adjusted to pH 7.4 with 1.0 M NaOH. A 0.1 M phosphate buffer was used as control solution. The dosage of the kynurenic acid solution was chosen on available data (Li and Smith 1997; Motekaitis et al. 1996). In our preliminary study, we found 1–2 nmol (0.1 M concentration) kynurenic acid solution was effective to attenuate the response of the DMNV neurons to electrical stimulation of the PVN, while 16 nmol reached the maximum effect that is comparable with dosages used by other investigators (Li and Smith 1997; Motekaitis et al. 1996; Sivarao et al. 1998). Therefore we tested the responses of the DMNV neurons to electrical stimulation of the PVN following injections of 4 nmol of kynurenic acid solution unless stated in the text. The duration of the effect of the kynurenic acid depends on the volume injected and was generally 20–60 min after administration. It was very difficult to test different dosages while recording a single neuron in vivo; therefore we were not able to generate a complete dosage curve.

To characterize the subtypes of the glutamate receptors, 15 mM [6-cyano-7-nitroquinoxaline-2,3-dione.2Na] (CNQX, Alexis Biochemicals, San Diego, CA) a potent and competitive AMPA receptor antagonist, was used to block AMPA receptor. The pharmacological properties of the antagonist has been fully characterized (Honore et al. 1988; Long et al. 1990; Turski et al. 1990). In addition, 15 mM DL-AP5 (A. G. Scientific, San Diego, CA), a potent and selective NMDA receptor antagonist (Davies et al. 1981; Hara et al. 1997; Watkins 2000) was injected to block NMDA receptor. The dosages of CNQX and DL-AP5 were modified from the published reports (Frigero et al. 2000; Li and Smith 1997). They were chosen at the dose that either completely abolished the responses of the DMNV neurons to electrical stimulation of the PVN or reached a maximum effect. Based on our preliminary study, we injected 1.2 nmol CNQX and/or DL-AP5 (15 mM in distilled water).

To exclude a possibility of passing fibers being activated during electrical stimulation of the PVN, we used 10 mM l-glutamate as a chemical stimulant that only acts on the cell body and not on passing fibers. After a neuron was characterized for response to electrical stimulation of the PVN, 0.8 nmol l-glutamate was injected into the PVN at the same area.

**RESULTS**

**Role of kynurenic acid on the excitatory influence of the PVN on DMNV neurons**

We tested the responses of 24 DMNV neurons excited by electrical stimulation of the PVN before and after injection of kynurenic acid into the DVC area near the recorded neurons. The DMNV neurons were characterized by having a fixed latency to electrical stimulation of the subdiaphragmatic vagal nerve, following high-frequency stimulation (75–150 Hz) and responding to a positive collision test. The neurons that did not meet these standards were intracellularly injected for later morphological verification or excluded. The general response of the 24 DMNV neurons excited by electrical stimulation of the PVN is summarized in Fig. 1A. The average basal rate of the 24 DMNV neurons was 1.33 ± 0.26 Hz. The firing rate increased to 3.43 ± 0.45 Hz (in period 2, an increase of 157.89%) and 3.55 ± 0.50 Hz (in period 3, 166.92% higher than the basal rate) in response to electrical stimulation of the PVN. The firing rate returned to 1.54 ± 0.34 Hz after the electrical stimulation of the PVN stopped; this is not significantly different from the prestimulation basal rate. The excitatory response of the DMNV neurons immediately followed the electrical stimulation of the PVN. Most of them returned to the basal firing rate after the stimulation stopped. The neuronal responses to electrical stimulation of the PVN with the same parameters were re-evaluated 2–5 min after injection of kynurenic acid into the DVC. Of the 24 DMNV neurons excited by the PVN stimulation, the glutamate receptor antagonist completely blocked the excitatory response of 22 DMNV neurons and significantly attenuated that of the other two DMNV neurons. The mean response of the 24 DMNV neurons to PVN stimulation after injection of the glutamate antagonist into the DVC is summarized in Fig. 1A’. The mean rate was 1.53 ± 0.34 Hz in period 1, 1.59 ± 0.32 Hz in period 2, and was 1.64 ± 0.32 Hz in period 3. As we have stated in METHODS, period 2 indicates the immediate responses and period 3 demonstrates the later responses to electrical stimulation of the PVN. Paired t-test reveals there is no difference between the periods 2 and 3 and period 1 (P = 0.079 and 0.303, respectively). B and C in Fig. 1 illustrate a sample of the DMNV neurons excited by electrical stimulation of the PVN and the effect of kynurenic acid on the excitatory influence of the PVN. The neuron was anterogradely excited by electrical stimulation of the PVN (Fig. 1, B and B’) and was retrogradely activated by electrical stimulation of the subdiaphragmatic vagal nerve. Injection of kynurenic acid solution into the DVC completely abolished both the spontaneous activity and the response to electrical stimulation of the PVN (period 1 in Fig. 1, C and C’). The basal rate and the response to the electrical stimulation of the PVN were recovered in 30 min after injection of kynurenic acid (Fig. 1, D and D’), indicating the reversibility of the blockage.

In addition to blocking the response of the DMNV neurons to electrical stimulation of the PVN, the average basal rate was
slightly higher after kynurenic acid injected into the DVC (from $1.33 \pm 0.26$ to $1.53 \pm 0.34$ Hz, *period 1* in Fig. 1, A and A'). The mean change was not significant compared with the basal rate before kynurenic acid application (P = 0.23 paired *t*-test). Although kynurenic acid did not affect the mean basal firing rate of the 24 DMNV neurons, many neurons did demonstrate a significant change in basal firing rates. However, the basal rate dropped significantly in 9 neurons and rose significantly in 14 DMNV neurons (an example of increased basal rate after the kynurenic acid injection can be seen in Fig. 2, D vs. C). The basal firing rate was unchanged by kynurenic acid in only one neuron. Although mean basal firing rates were not
significantly different between the two groups ($P > 0.05$), there was a significant difference in the basal firing rates between them ($P < 0.01$) after kynurenic acid injection. The basal rate of the nine neurons decreased from $1.64 \pm 0.53$ Hz to $0.40 \pm 0.20$ Hz ($P < 0.05$) after kynurenic acid injection. In contrast, the glutamate antagonist raised the spontaneous rate of the 14 DMNV neurons from $1.23 \pm 0.36$ to $2.62 \pm 0.45$ Hz ($P < 0.01$).
Excitatory influence of the PVN on nine DMNV neurons were reversed by the glutamate antagonist

Among the 24 DMNV neurons that were excited by electrical stimulation of the PVN before application of the glutamate antagonist, nine of them (37.5%) were actually inhibited slightly but significantly by electrical stimulation of the PVN after kynurenic acid was injected into the DVC. Figure 2 shows one example of these neurons. The neuron was inhibited by gastric (Fig. 2, A and A’) and intestinal (Fig. 2, B and B’) distention. Electrical stimulation of the PVN significantly increased the firing rate of the neuron (Fig. 2C and C’). After injection of 4 nmol kynurenic acid into the DVC, the excitatory response to electrical stimulation of the PVN was completely blocked. Furthermore, electrical stimulation of the PVN with the same parameters actually inhibited the firing rate of the neuron (Fig. 2, D and D’). The significance of this inhibition was shown by comparing periods 2 and 3 with prestimulation period 1 (P < 0.001 and P = 0.013 respectively, ANOVA multiple comparison with post hoc Bonferroni’s test). The excitatory response to electrical stimulation recovered 30 min after injection of the glutamate antagonist (Fig. 2, E and E’).

As we have mentioned previously, most of excitatory responses of the DMNV neurons to the PVN activation were completely blocked or reversed by the glutamate antagonist. However, 2 of the 24 neurons demonstrated a different response property (see Fig. 3 for a sample). The neuron was excited by electrical stimulation of the PVN with robust excitation for a short time then inhibited for the rest of the period of the stimulation (Fig. 3, A and A’). The excitatory response to electrical stimulation of the PVN was attenuated by injection of 8 nmol kynurenic acid but was still significantly higher than the basal rate. Another 8 nmol kynurenic acid decreased the basal firing rate further, but the neuron still activated slightly on electrical stimulation of the PVN (Fig. 3, B and B’). In this case, the inhibitory period following excitation in the period 3 was also abolished by kynurenic acid. Variable latency (38–63) to repeated electrical stimulation of the PVN indicates synaptic action potentials (Fig. 3C, 10 sweeps). The neuron was retrogradely activated by 100-Hz electrical stimulation of the subdiaphragmatic vagal nerve with an 82-ms fixed latency (Fig. 3D).

**Effect of AMPA receptor antagonist**

To elucidate the subtype of the glutamate receptors involved in the response, we characterized the effects of the selective...
AMPA receptor antagonist CNQX. In 6 of 12 DMNV neurons, excitation elicited by electrical stimulation of the PVN was completely blocked and in 3 of the 6 DMNV neurons, the response to the PVN stimulation was reversed to be inhibitory after administration of CNQX (15 mM concentration, 1.2 nmol). In the other six neurons, CNQX greatly attenuated the excitatory influence of electrical stimulation of the PVN. Figure 4A shows the average response of the 12

FIG. 4. The histograms show mean response profiles of the DMNV neurons that responded to electrical stimulation of PVN before and after injection of glutamate antagonists. Horizontal lines above the drawings indicate the duration of electrical stimulation of the PVN. The histograms show average rate per 5 s. A: general responses of 12 DMNV neurons to electrical stimulation of the PVN. B: the response of 5 DMNV neurons to electrical stimulation of the PVN. C: the mean responses of 11 DMNV neurons to electrical stimulation of the PVN. B: these changes were abolished by CNQX + DL-AP5. C: the response profile of 11 DMNV neurons after injection DL-AP5, an NMDA receptor antagonist.
DMNV neurons to electrical stimulation of the PVN (from 1.31 ± 0.40 to 3.99 ± 0.81 Hz, \( P < 0.05 \)) and \( A' \) shows the mean change after injection of the CNQX solution into the DVC area (from 2.33 ± 0.79 to 2.94 ± 0.75 Hz, \( P > 0.05 \)).

In addition, the basal firing rate of seven DMNV neurons was significantly raised, that of two DMNV neurons was significantly decreased, and that of the remaining three DMNV neurons was not changed. However, the change of the mean basal rate of the 12 DMNV neurons was not significant (from 1.31 ± 0.40 to 2.33 ± 0.79 Hz, \( P = 0.114 \)).

**Role of NMDA receptor antagonist**

Although CNQX can block the excitatory responses of 6/12 DMNV neurons to electrical stimulation of the PVN, the other 6 of 12 DMNV neurons were still slightly excited by electrical stimulation of the PVN after injection of CNQX. To test whether this residual excitation is mediated by an NMDA receptor, we further injected \( \alpha \)-AP5 (15 mM concentration, 1.2 nmol) in five of the six DMNV neurons. The results indicate that \( \alpha \)-AP5 further attenuated the residual excitations (Fig. 4, \( B \) and \( B' \)). Figure 5 shows a sample of these neurons. The neuron was firing at a low basal rate and was dramatically excited by electrical stimulation of the PVN (0.2 mA, 1-ms duration and 15 Hz; \( A \) and \( A' \)). Injection of 1.2 nmol CNQX (selective AMPA receptor antagonist) almost completely blocked the excitatory influence of electrical stimulation of the PVN (\( B \) and \( B' \)) and slightly raised the basal firing rate (from 0.23 to 0.63 Hz, \( P < 0.001 \)). An additional 1.2 nmol \( \alpha \)-AP5 (specific NMDA receptor antagonist) reversed the response of the DMNV to electrical stimulation of the PVN (\( C \) and \( C' \)). The response to electrical stimulation of the PVN partially recovered 25 min after the CNQX and \( \alpha \)-AP5 injections (\( D \) and \( D' \)).

**FIG. 5.** The drawings illustrate the response properties of a DMNV neuron to electrical stimulation of the PVN. The neuron was firing at a low basal rate and was dramatically excited by electrical stimulation of the PVN (0.2 mA, 1-ms duration and 15 Hz; \( A \) and \( A' \)). Injection of 1.2 nmol CNQX (selective AMPA receptor antagonist) almost completely blocked the excitatory influence of electrical stimulation of the PVN (\( B \) and \( B' \)) and slightly raised the basal firing rate (from 0.23 to 0.63 Hz, \( P < 0.001 \)). An additional 1.2 nmol \( \alpha \)-AP5 (specific NMDA receptor antagonist) reversed the response of the DMNV to electrical stimulation of the PVN (\( C \) and \( C' \)). The response to electrical stimulation of the PVN partially recovered 25 min after the CNQX and \( \alpha \)-AP5 injections (\( D \) and \( D' \)).
excited by 0.2 mA electrical stimulation of the PVN (Fig. 5, A and A’). Injection of 1.2 nmol CNQX almost completely blocked the excitatory influence of electrical stimulation of the PVN (Fig. 5, B and B’) as well as slightly raised the basal firing rate (from 0.23 to 0.63 Hz, \( P < 0.001 \)). An additional 1.2 nmol DL-AP5 reversed the response of the DMNV neuron to electrical stimulation of the PVN (Fig. 5, C and C’). The response to electrical stimulation of the PVN partially recovered 25 min after CNQX and DL-AP5 (Fig. 5, D and D’).

In contrast, DL-AP5 alone had minor effects on the excitation of the DMNV neurons induced by electrical stimulation of the PVN. Among 11 DMNV neurons that were excited by electrical stimulation of the PVN, DL-AP5 only partially but significantly attenuated the excitatory responses of 4 DMNV neurons to electrical stimulation of the PVN and did not completely block any of them. The average responses of the 11 DMNV neurons to electrical stimulation of the PVN before and after DL-AP5 are illustrated in Fig. 4, C and C’. Electrical stimulation of the PVN significantly increased the basal rate of the 11 DMNV neurons from 1.30 ± 0.30 to 3.80 ± 0.87 Hz (\( P < 0.01 \), Fig. 4C). After injection of DL-AP5, PVN stimulation still significantly raised the basal rate from and from 1.20 ± 0.29 to 2.91 ± 0.46 Hz (\( P < 0.01 \), Fig. 4C’). The mean basal rates of the 11 DMNV neurons before and after DL-AP5 were not significantly different (1.30 ± 0.30 and 1.20 ± 0.29 Hz, respectively, \( P > 0.05 \)).

**Chemical stimulation of the PVN**

In the present experiment, to prove the electrical stimulation of the PVN activated the PVN neurons, we injected glutamate into the PVN where the stimulating electrode was located. We placed the stimulating electrode in the PVN as well as a multibarreled pipette into the same place. Thus we were able to activate the same group of the PVN neurons by both electrical and chemical stimulation. In seven DMNV neurons that were excited by electrical stimulation of the PVN, five neurons were also excited by injection of 0.8 nmol l-glutamate into the PVN. The excitatory response of the DMNV neurons to the chemical stimulation of the PVN had 5–10 s latency and lasted 2–3 min. Electrical stimulation of the PVN increased the mean activity of the seven DMNV neurons from 0.75 ± 0.39 to 2.41 ± 0.87 Hz (\( P < 0.05 \), paired \( t \)-test). Glutamate injected into the PVN raised the mean basal firing of the seven neurons from 0.79 ± 0.43 to 1.48 ± 0.64 Hz (\( P < 0.05 \)). A sample of combined electrical and chemical stimulation of the PVN is illustrated in Fig. 6. The neuron was retrogradely activated by electrical stimulation of the subdiaphragmatic vagal nerve. It was completely inhibited by gastric distention and unresponsive to intestinal distention. The neuron was slightly excited by electrical stimulation of the PVN (Fig. 6, A and A’, \( P = 0.001 \)). Injection of 0.8 nmol l-glutamate into the PVN significantly excited the neuron (Fig. 6, B and B’, \( P < 0.001 \), ANOVA Bonferroni’s test). The results indicate that the excitatory influence of the PVN on the DMNV neurons was mainly from the PVN neurons.

**Synaptic activation of the DMNV neurons by electrical stimulation of the PVN**

As we have stated previously, electrical stimulation of the PVN may excite a group of the gut-related DMNV neurons. In the present experiment, we further characterized the electrophysiological property of the responses of the DMNV neurons to electrical stimulation of the PVN. The location of the stimulating electrode was verified by histology to be within the boundary of the PVN. A sample of the electrode location in the PVN is illustrated in Fig. 7. The PVN is outlined by a dotted line and the electrode tip was in the parvocellular part of the PVN. To confirm the neurons in the DVC, as we have mentioned in the method section, 10 neurons were intracellular injected to verify the property of the neurons. Figure 8 illustrates a sample of these neurons. Histochemistry demonstrated the neuron’s morphology and its relationship with nearby neurons.
structure. The neuron was located in the medial part of the DMNV, 100 μm caudal to the obex. There are three major dendrites directed to the Medial NST, subpostremal NST and the contralateral NST. A single axon arises from the root of one major dendrite and runs ventrolaterally to exit the brain stem (Fig. 8A). The neuron was excited by electrical stimulation of the PVN (Fig. 8B) and retrogradely activated by stimulation of the subdiaphragmatic vagus nerve with a constant 105-ms latency (Fig. 8C), indicating the neuron projects to the abdominal organs.

In addition, we characterized the latencies of 14 DMNV neurons to electrical stimulation of the PVN. The results revealed that the mean latency was 37.71 ± 2.40 ms. Multiple responses of the action potentials to a single electrical stimulation of the PVN were observed in six of the DMNV neurons. Figure 9A shows a DMNV neuron that responded to electrical stimulation of the PVN with multiple action potentials. The DMNV neuron followed 3-Hz current stimulation of the PVN. A single electrical stimulation (indicated by solid arrows) induced multiple action potentials with a latency of 40–45 ms (2–3 action potentials for each stimulus, indicated by the empty arrows). In all three DMNV neurons that were recorded intra-
cellularly, monosynaptic action potentials and excitatory postsynaptic potentials in response to electrical stimulation of the PVN were characterized. A monosynaptic activation of the DMNV neurons is illustrated in the Fig. 9B. The neuron was intracellularly recorded. Electrical stimulation of the PVN elicited either full-sized action potentials (empty arrows) or excitatory postsynaptic potentials (EPSP). C: the monosynaptic excitatory postsynaptic potentials following electrical stimulation of the PVN (50-sweep overlaps). The neuron was recorded when a negative current was injected to inhibit the excitability of the neuron. The y axes indicate the membrane potentials and x axes demonstrate time. The scales have been marked on the all axes.

DISCUSSION

PVN may excite and inhibit DMNV neurons

In a previous study, we have reported ~80% of the DMNV neurons responded to electrical stimulation of the PVN. The majority (59%) of the neurons that responded to electrical
stimulation of the PVN was excited by the PVN stimulation. PVN activation seems to have an opposite effect to the gastrointestinal distention on the DMNV and NST neurons. Although the dominant role of electrical stimulation of the PVN is excitatory on the DMNV neurons, there are two subsets of neurons: one excited and the other inhibited by the PVN (Banks and Harris 1987; Nishimura and Oomura 1987; Zhang et al. 1999). In addition, the two opposite influences on the DVC neurons by the PVN have been indicated by some other physiological studies (Rogers and Hermann 1986, 1987; van Dijk et al. 1994; Shiraishi 1988). The possible explanation for these disparate results is that they may activate different subsets of the PVN neurons.

The diverse influence of the PVN on vagal neuron activities provides one explanation of the opposite results that have been obtained regarding PVN activation for both excitatory and inhibitory influences on gastrointestinal functions such as gastric acid secretion (Rogers and Hermann 1986; Shiraishi 1988; Yoneda and Tache 1995), gastric motility (Rogers and Hermann 1987), and parasympathetic activity (van Dijk et al. 1994). A detailed discussion of the potential role of the descending PVN–DMNV pathway as well as the interaction of PVN–DMNV pathway with the gastrointestinal stimuli can be found in our previous publication (Zhang et al. 1999).

In addition to the regulatory role of the PVN on DMNV neurons, a growing body of evidence is revealing the importance of the PVN-DMNV on appetite, satiety, and feeding behavior (Rogers et al. 1996). Destruction of the ventromedial hypothalamus (VMH), a structure immediately ventral to the hypothalamus (VMH), a structure immediately ventral to the hypothalamus, produced obesity represented by hyperphagia, hyperinsulinemia, and hypertriglyceridemia. Experiments show that, following lesion of the hypothalamus, the vagotomized animals failed to overeat or gain excessive weight on a standard laboratory diet. This indicates hypothalamic obesity requires the integrity of the vagus (Bray 1985). We would like to point out that the VMH obesity might be closely related to the PVN-DMNV pathway. Unlike the extensive connections between the PVN and DVC, there are few connections between the VMH and the DVC. Because the VMH obesity is vagally dependent, the influence of the VMH lesion on the vagus may be mediated by the PVN. Given the close proximity of the VMH and PVN, another possibility is that the VMH lesion may simultaneously injure the PVN. This argument is supported by an observation reported by Cox and Sims: simultaneously damaging one side of the VMH and the contralateral PVN produced significant hyperphagia and weight gains not significantly different from that in rats with bilateral lesions of the VMH or the PVN. The results suggest that VMH lesion induced obesity may be the result of damaging efferent projections of the PVN neurons that run through the VMH (Cox and Sims 1988).

**PVN may have a direct influence on DMNV neurons**

When stimulating central areas with electrical current, one must always be aware of the nonselective property of electrical stimulation. It will activate any neuronal structures: neurons or passing fibers that originate far from the point of stimulation. We used electrical stimulation of the PVN and selective antagonists to characterize the putative neurotransmitter that mediates the excitatory influence of the PVN on the gut-sensitive DMNV neurons. There is always the possibility that electrical stimuli in the hypothalamus are not limited to the PVN. However, indirect evidence suggests that the electrical stimulation mainly activated the PVN. Anatomical data have shown that the PVN has extensive projections to the DVC, whereas the projections from the other parts of the medial hypothalamus and perifornical area are scarce (Holstege 1987; Horny and Piekut 1988; Loewy 1991; van der Kooy et al. 1984; Willett et al. 1987). In addition, although the electrical stimulation of the PVN may be indirectly exciting the DMNV neurons by affecting the NST neurons, it has been reported that the PVN projects primarily to the DMNV, whereas its projections to the NST are lighter or free of labels (Saper et al. 1976; Willett et al. 1987). This is in accordance with our previous investigation where we found that more neurons and stronger responses were recorded in the DMNV than in the NST (Zhang et al. 1999).

Banks and Harris reported that DMNV neurons were synaptically activated following electrical stimulation of the PVN (Banks and Harris 1987). Our data also show that electrical stimulation of the PVN may monosynaptically activate the DMNV neurons (see Fig. 9, B and C, for an example), which was also reported by Nishimura and Oomura (1987).

Another side effect of electrical stimulation of the PVN is activation of the passing fibers. To our knowledge, projecting fibers from other central areas that pass the PVN to the DVC have not been reported. In addition, we demonstrate that chemical stimulation of the PVN with glutamate, which excites the neurons but not the passing axons, was able to excite the DMNV neurons. The results indicate that the electrical stimulation is in accordance with the chemical stimulation. All these findings indicate that the PVN has a direct influence on the DMNV neurons. However, we must take into consideration of the limits of the electrical and chemical stimulation. Neither of them could exclude the involvement of the neurons near the PVN. Fortunately, these neurons rarely project to the DVC suggesting less possibility of direct involvement of these neurons.

We noticed that a single electrical stimulation induced multiple action potentials. We postulate that a single DMNV neuron receives more than one excitatory afferents from the PVN. Because these afferents have slightly variation of the latency, a single electrical stimulation of the PVN will be able to induce more than one action potential. However, in an intracellular observation, the EPSPs are longer than an action potential, so multiple EPSPs would overlap and become inseparable. Another explanation is that a single electrical stimulation may release enough neurotransmitter to elicit action potentials more than once.

Glutamate mediates the excitatory influence of the PVN on DMNV neurons

As we have reported previously, the PVN has two concurrent influences on the DMNV neurons. The possible excitatory neurotransmitters remained to be identified. In the present study, our data suggest that glutamate released from the PVN is responsible for excitation of the DMNV neurons. We found that the excitatory effect on the DMNV neurons was almost 100% blocked by the glutamate antagonists. This result indicates that glutamate is the major (if not the only) excitatory neurotransmitter in the DMNV when the PVN is activated. The
result is contrary to the current understanding about the PVN-DVC interaction. Immunohistochemical staining combined with retrograde tract tracing methods shows that PVN neurons containing oxytocin, vasopressin, somatostatin, and enkephalin project to the DVC (Sawchenko and Swanson 1982). However, while no glutamate-containing PVN neurons have been reported projecting directly to the DVC area, to our knowledge, only ~25% of the long projecting PVN to DVC neurons have been identified as containing recognized neurotransmitters. This leaves a large proportion of the projecting PVN neurons unidentified. In addition, accumulating data indicate that glutamate may participate in the interaction in the axis of the PVN-DVC. Data have shown that glutamate is a ubiquitous neurotransmitter in the CNS (Watkins 2000) and glutamate-containing neurons have been localized in the PVN by immunocytochemistry (Matsumoto et al. 1994) as well as electrophysiology (Daftary et al. 1998).

**PVN modulates DMNV neurons**

The physiological effect of the PVN on the DMNV neurons is not very clear. Behavioral studies suggest the PVN may have a tonic inhibition of the vagus (Flanagan et al. 1992). VMH weighting appears to be associated with an increased vagal efferent tone together with decreased sympathetic efferent activities (Bray 1985; Inoue and Bray 1977). The electrophysiological results indicating that the DMNV neurons excited by electrical stimulation of the PVN outnumbered those inhibited (Zhang et al. 1999) seem to conflict with the results from the behavior studies. Our present study may partially answer the question. As we have mentioned in Results, the average of the basal activity of the DMNV neurons before kynurenic acid injection was not significantly different from that after kynurenic acid injection (Fig. 1). However, the basal firing rates of 21 of 24 neurons changed significantly (decreased or increased) following injection of kynurenic acid. There are eight DMNV neurons (33%) decreased basal rates after injection of the glutamate antagonist. For example, injection of kynurenic acid solution completely abolished both the basal firing and the response to the PVN activation of the neuron illustrated in Fig. 1. C and C’ (see next paragraph for possible explanation for the increased basal rate in 13 DMNV neurons). It is reasonable to postulate that the neurons with decreasing basal rates in the presence of the glutamate antagonist may be under tonic excitation of the PVN. We postulate that there are two groups of projecting PVN neurons: one has inhibitory and the other has excitatory influences on the DMNV neurons (each group may contain >1 subgroup). It is worth to point out that the more active one of the two groups needs to be identified. Extracellular recording of the PVN neurons projecting to the DVC reveals that these neurons discharge at a lower rate and showed no phasic firing patterns (Kannan and Yamashita 1983). Additionally, ~50% of the neurons are silent (Bagdan and Pittman 1995). These data suggest electrophysiological heterogeneity of the PVN projecting neurons. Given the fact that some neurons are tonically active and some are not in physiological condition, the tonic firing neurons may be those inhibiting the DMNV. When these neurons are damaged or deactivated, the DMNV neurons will be more active and gastrointestinal motility, and acid and insulin secretions will increase accordingly as seen in lesions of the ventromedial hypothalamus (Albrecht et al. 1988) and the PVN (Tokunaga et al. 1986a). In other words, electrical or chemical stimulation of the PVN may have a stronger effect on the PVN neurons that excite the DMNV. This hypothesis suggests an important mechanism of the PVN in the regulation of feeding behavior that involves a complex circuit and many feeding-related neuronal transmitters such as leptin, neuropeptide Y, galanin, and norepinephrine (Tritos and Maratos-Flier 1999).

Data have shown that there is more than one population of DMNV neurons according to electrical stimulation of the PVN (Banks and Harris 1987; Zhang et al. 1999). In the present study, ~37% of recorded DMNV neurons switched their response to PVN stimulation from excitatory to inhibitory. One possible explanation is that a single neuron from a specific group of the DMNV neurons receives both excitatory and inhibitory inputs from the PVN (and perhaps other neural centers). In this case, electrical stimulation of the PVN excites both excitatory and inhibitory afferents to the DMNV neuron. In the normal condition, the excitatory response is dominant in those neurons showing an excitatory response to electrical stimulation of the PVN. Equivalently, those neurons that receive a dominant inhibitory input from the PVN will demonstrate an inhibitory response to electrical stimulation of the PVN. In a specific condition, this balance may be interrupted or reversed. For example, in the present study, those neurons that receive both excitatory and inhibitory inputs will show inhibitory response to electrical stimulation of the PVN after the excitatory input is blocked by the specific antagonist. Of course, it is unlikely that complete blocking of either the excitatory or the inhibitory input to a neuron is achieved in the physiological condition. However, any changes out of balance may ultimately lead to a pathological condition.

In the present study, 14 of the 24 DMNV neurons increased their basal firing rate after application of the glutamate antagonist (see Fig. 2, D vs. C, in period 1 for a sample). The mechanisms are not clear. However, glutamate is found as an excitatory neurotransmitter in the DVC not only from the PVN but also from vagal afferents (Saha et al. 1995a,b). The inhibitory response of DMNV neurons to gastrointestinal stimuli (Fogel et al. 1996; Zhang et al. 1992, 1995, 1998) may be indirectly mediated by vagal afferents containing glutamate through a mechanism involving GABA receptors (Hornby 2001). In addition to blocking the excitatory input from the PVN, the glutamate antagonist injected into the DVC also eliminated tonic inhibition from vagal afferents. Removal of tonic inhibition from vagal afferents might lead to an increased basal activity in some DMNV neurons.

Although the results indicate that the AMPA receptor plays a major role in the excitatory response of the DMNV neuron, the NMDA receptor is also involved in the reflex. For example, Dl-AP5 eliminated residual excitatory response to electrical stimulation of the PVN in six DMNV neurons that have been greatly attenuated by the AMPA receptor antagonist (see Fig. 5, B and C, for a sample). Among 11 DMNV neurons, Dl-AP5 partially but significantly attenuated the excitatory responses of four DMNV neurons to electrical stimulation of the PVN. It is possible that two groups of the DMNV neurons are present. One group expresses only the AMPA subtype of the glutamate receptor, while the other contains both AMPA and NMDA receptor subtypes. This hypothesis is partially supported by a report indicating that both AMPA and NMDA receptors are
present in DMNV neurons (Travaglì et al. 1991). In addition, functional and anatomical heterogeneity of DMNV neurons has been indicated (Fogel et al. 1996; Krowicki et al. 1997; Manier et al. 1990; Zhang et al. 1995, 2000). However, additional histochemical data will be needed to clarify this argument.

**Technical considerations**

In the present study, we used electrical and chemical stimulation of the PVN, microelectrode recording, and local microinjection of the glutamate receptor antagonists. The pitfall of the electrical and chemical stimulation of the PVN has been discussed in the previous section and previous publication (Zhang et al. 1999).

As to the techniques in the gastrointestinal distention, we have successfully used this technique for several investigations. The results indicate that the technique is reliable and replicable. We used this technique as our standard to compare results from different projects (Fogel et al. 1996; Renehan et al. 1995; Zhang et al. 1992, 1995, 1999).

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