Hypothalamus–Brain Stem Circuitry Responsible for Vagal Efferent Signaling to the Pancreas Evoked By Hypoglycemia in Rat

Xiaoyin Wu, Jun Gao, Jin Yan, Chung Owyang, and Ying Li
Gastroenterology Research Unit, Department of Internal Medicine, University of Michigan Health System, Ann Arbor, Michigan 48109

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Wu, Xiaoyin, Jun Gao, Jin Yan, Chung Owyang, and Ying Li. Hypothalamic–brain stem circuitry responsible for vagal efferent signaling to the pancreas evoked by hypoglycemia in rat. J Neurophysiol 91: 1734–1747, 2004. First published November 26, 2003; 10.1152/jn.00791.2003. Circulating glucose levels significantly affect vagal neural activity, which is important in the regulation of pancreatic functions. Little is known about the mechanisms involved. This study investigates the neural pathways responsible for hypoglycemia-induced vagal efferent signaling to the pancreas and identifies the neurotransmitters involved. Vagal pancreatic efferent nerve activities were recorded in anesthetized rats. Insulin-induced hypoglycemia, a decrease of blood glucose levels from 114 ± 5 to 74 ± 6 mg dl⁻¹, stimulated an increase in pancreatic efferent nerve firing from a basal rate of 1.1 ± 0.3 to 19 ± 3 impulses 30 s⁻¹. In contrast, vagal primary afferent nerve discharges recorded on the nodose ganglia were unaltered by systemic hypoglycemia. Vagal afferent rootlet section plus splanchinectomy had no effect on hypoglycemia-induced vagal efferent firing, suggesting a central site of action. Decerebration reduced the increase in nerve firing stimulated by hypoglycemia from 21 ± 4 to 9.6 ± 2 impulses 30 s⁻¹. Chemical ablation of the lateral hypothalamic area, but not the arcuate nucleus, inhibited pancreatic nerve firing evoked by hypoglycemia. Microinjection of the orexin-A receptor antagonist SB-334867 into the dorsal motor nucleus of the vagus (DMV) inhibited pancreatic nerve firing evoked by insulin-induced hypoglycemia by 56%. In contrast, injection of orexin-A (20 pmol) into the DMV elicited a 30-fold increase in pancreatic nerve firing. We concluded that systemic hypoglycemia stimulates pancreatic efferent nerve firing through a central mechanism. Full expression of pancreatic nerve activities during hypoglycemia requires both the forebrain and the brain stem. In addition to activating neurons in the brain stem, central neuroglucopenia activates subpopulations of neurons in the lateral hypothalamic area that contain orexin. The released orexin acts on DMV neurons to stimulate pancreatic efferent nerve activities and thus regulate pancreatic functions.

INTRODUCTION

Glucose, which is essential for brain energy metabolism, must be derived from the circulation. Despite wide variations in glucose flux (e.g., during a fast, after a meal, and while exercising), the plasma glucose concentration is normally tightly regulated. Such is not the case for individuals taking drugs that lower the plasma glucose concentration. Hypoglycemia is a fact of life for people with type 1 (insulin-dependent) diabetes mellitus, who typically suffer countless episodes of symptomatic hypoglycemia over a lifetime of diabetes (Cryer 1997). Insulin-induced hypoglycemia traditionally has been used as an experimental tool to stimulate vagally medi- ated gastric acid and pancreatic enzyme secretion (Rosenberg et al. 1976; Smith et al. 1981; Wettergren et al. 1998). The autonomic nervous system plays an important role in regulating the glucagon response to insulin-induced hypoglycemia (Taborsky 2001). Niijima (1975) reported that hypoglycemia decreases pancreatic vagal nerve activity in rabbits, contradicting a great body of evidence that insulin-induced hypoglycemia stimulates gastric acid, bile, and pancreatic enzyme secretions. The plasma pancreatic polypeptide response, which is stimulated by hypoglycemia or food intake, is an index of vagal cholinergic input to the pancreas. Currently, the neural pathways responsible for mediating pancreatic nerve activities during glucoprivation remain an enigma.

The pancreas receives parasympathetic innervation from preganglionic neurons in the dorsal motor nucleus of the vagus (DMV). Our research has provided functional and electrophysiological evidence that neuropeptide Y (NPY) and substance P stimulate, whereas somatostatin and calcitonin gene–related peptide inhibit, the DMV neurons that project to the pancreas (Li and Owyang 1993a; Li et al. 1998; Wu et al. 2001, 2002). These pathways are responsible for mediating pancreatic enzyme secretion stimulated by meal-related luminal factors (Li and Owyang 1993b; Li et al. 2001). Numerous classic neuroanatomical and neurophysiological studies have shown that the lateral hypothalamic area (LHA) modulates the efferent output of pancreatic nerves (Bernardis and Bellinger 1996; Buijs et al. 2001; Williams et al. 2001). Immunocytochemistry studies have confirmed the presence of the neuropeptide orexin in the projections of the LHA neurons to the DMV (Buijs et al. 2001). Fos-like immunoreactivity during insulin-induced hypoglycemia was evident in 30% of the orexin-immunoreactive neurons (Cai et al. 2001). We hypothesize that systemic hypoglycemia stimulates vagal efferent signaling to the pancreas by a central site of action. Orexin released from the LHA acts on vagal preganglionic motor neurons to stimulate pancreatic efferent nerve firing.

In this study, we performed electrophysiological studies in rats to investigate the vagal pancreatic efferent nerve responses to insulin-induced hypoglycemia. We recorded pancreatic nerve responses to central hypoglycemia induced by intracebroventricular (i.c.v.) administration of an antimetabolic glucose analog, 5-thioglycolate (5TG). To determine the sensitivity of vagal primary afferent neurons to hypoglycemia, we examined the effects of hypoglycemia on vagal nodose neuronal activities. We studied the contribution of the hindbrain and forebrain in the modulation of pancreatic nerve firings acti-
vated by systemic hypoglycemia in decerebrate rats. To characterize the hypothalamus-brain stem circuitry responsible for vagal efferent signaling, the effects of bilateral chemical lesions of the LHA on pancreatic nerve discharges stimulated by insulin-induced hypoglycemia were recorded. The role of central endogenous orexin in the modulation of pancreatic nerve responses was also studied.

METHODS

Materials

Orexin, insulin, and 5TG were purchased from Sigma-Aldrich (St. Louis, MO). The NPY receptor antagonist [d-Try^{27,36},d-Thr^{32}]NPy27-36 was purchased from Research Biochemical International (Natick, MA). The orexin-A receptor antagonist SB-334867 was provided by GlaxoSmithKline (Research Triangle Park, NC).

Animal preparation

All protocols were approved by the Committee for the Use and Care of Animals at the University of Michigan. After an overnight fast, male Sprague-Dawley rats weighing 250 to 300 g were anesthetized with an intramuscular injection of xylazine and ketamine (13 and 87 mg kg$^{-1}$ body wt, respectively). Ketamine reportedly has little or no effect on blood glucose levels (Aynsley-Green et al. 1973). Supplemental doses of these agents were administered as required to maintain a deep level of anesthesia and muscle relaxation. Polyethylene catheters were placed in the external jugular vein and tail vein for intravenous infusion of insulin or glucose. At the end of the study, the animals were killed with an overdose of ketamine. Carcasses were placed in a carcass disposal barrel, which was picked up weekly for incineration.

Electrophysiological recording of vagal pancreatic efferent nerve activity

A tracheal tube was inserted to permit artificial ventilation with room air (75 to 85 strokes min$^{-1}$, 3.5 to 4.0 cm$^3$ tidal vol). Body temperature was maintained with a special heating pad. A branch of the vagus nerve to the splenic artery innervating the pancreas was isolated from the central cut end. The efferent nerve activity was recorded in the central cut end using bipolar platinum electrodes. A strip of connective tissue was wrapped around the second indifferent electrode. Thus multiunit efferent recordings were obtained from the vagal pancreatic nerve (Li et al. 2003). The electrophysiological signal was amplified by an AM system high-input impedance that had been preamplified, and monitored with an oscilloscope and audio monitor. The discharges were displayed and stored electronically using Axotape software (Axon Instruments, Union City, CA) and a 160-MHz Pentium processor, and subsequently analyzed off-line. The stability of the firing frequency was confirmed by recording vagal pancreatic nerve firing for 5 min, and then monitoring basal discharge for 5 min. Deep anesthesia and the absence of electrocardiographic and electromyographic interferences attributed to the proximity of the reference electrode to the recording electrode prevented movement artifacts.

Recording of nodose neuronal activity

Rats were placed in a small animal stereotaxic instrument (David Kopf Instruments, Tujunga, CA). The right nodose ganglion was exposed using a short dorsal approach. Using an operating microscope, the ganglion sheath was separated and removed from the adjacent cervical sympathetic trunk and carotid artery. The discharges of the vagal primary afferent neurons supplying the gastrointestinal tract were recorded from the nodose ganglion by means of extracellular glass-coated tungsten microelectrodes, as previously described (Li et al. 1999; Zhu et al. 2001).

Experimental design

STEEPED HYPOGLYCEMIC CLAMP EXPERIMENTS. A modified hyperinsulinemic glucose clamp technique was used to maintain the blood glucose at predetermined levels (Nauck et al. 2002). After obtaining basal blood samples, intravenous infusion of porcine insulin (2.0 mU kg$^{-1}$ min$^{-1}$; Eli Lilly, Indianapolis, IN) was begun. Similar to previous studies in humans (Nauck et al. 2002) and dogs (Jackson et al. 2000), our preliminary results indicated that the lower glycemic plateau could be reached without increasing the insulin infusion rate. Blood glucose was initially measured every 3 min until a stable level was achieved, and then at 6-min intervals. Capillary samples (approximately 100 μL) drawn from tail to ensure glucose concentrations close to those of arterial plasma. The blood glucose concentration was measured using a glucose oxidase method with the YSI 2300 STAT Plus glucose analyzer (YSI Incorporated, Yellow Springs, OH). For some of the studies, we also used a glucometer (OneTouch II, LifeScan, Milpitas, CA). Consistent with previous reports (Junghem and Koschinsky 2002; Planche et al. 2001), we found that the results from the OneTouch glucometer were in good agreement with the YSI 2300 reference meter. Insulin was measured using an insulin microparticle enzyme immunoassay (Chemistry Laboratory of the Michigan Diabetes Research and Training Center). Counterregulatory hormone concentrations were not estimated. When necessary, glucose (20% dextrose; Baxter, Deerfield, IL) was infused at a rate that would provide the desired blood glucose concentration to maintain the hypoglycemic clamp. The glucose concentration was allowed to fall slowly to the desired range during the initial 15 min, and was maintained at that range during the 30-min recording period. The glucose infusion rates and the times when those rates changed were recorded to facilitate calculation of the total amount of glucose infused to maintain glucose levels in the desired range. Firing of the pancreatic efferent nerve during insulin-induced hypoglycemia was recorded at 3 different blood glucose levels: 86 ± 4, 74 ± 6, and 57 ± 5 mg dl$^{-1}$ (basal: 114 ± 5 mg dl$^{-1}$). In the preliminary studies, nerve recording experiments could usually be completed at 2 desired glucose levels for each rat. To determine whether systemic hypoglycemia acts on the vagal afferent pathway, nodose ganglia recordings were performed during insulin-induced hypoglycemia in a separate group of rats.

VAGAL AFFERENT ROOTLET SECTION AND SPLANCHNICTOMY.

To identify the central or peripheral sites of glucose-sensing neurons responsible for modulating pancreatic efferent nerve firing activated by hypoglycemia, we performed vagal afferent rootlet section plus splanchnictomy. In the rat, selective afferent rootlet section is possible because the afferent rootlet enters the medulla dorsal to the exit site of the efferent rootlet. This technique was previously validated by a study that examined the effects of CCK octapeptide (CCK-8) on pancreatic responses (Li and Owyang 1993b). Physiological doses of CCK-8 completely abolished pancreatic responses but supraphysiological doses had no effect. The splanchnic nerve bundle projecting from the site of the efferent rootlet. This technique was previously validated by a study that examined the effects of CCK octapeptide (CCK-8) on pancreatic responses (Li and Owyang 1993b). Physiological doses of CCK-8 completely abolished pancreatic responses but supraphysiological doses had no effect. The splanchnic nerve bundle projecting to the pancreas was cut around the left and right gastric artery at the level of the adrenal gland. Vagal pancreatic efferent nerve discharge in response to insulin-induced hypoglycemia was measured 1 h after surgery.

I.C.V. ADMINISTRATION OF AN ANTIMITABOLIC GLUCOSE ANALOG, 5TG.

To determine the direct effect of central hypoglycemia on vagal pancreatic efferent nerve firing, we used an in vivo anesthetized rat model prepared with a guide cannula inserted into the middle of the left cerebral ventricle. The cannula was secured by 2 screws inserted into the surface of the parietal bone and reinforced using cranioplastc plastic (Plastics One, Wallingford, CT). Coordinates from the bregma were as follows: anteroposterior, 0.6 mm; lateral, 2 mm; ventral, 4 mm (Waynfforth 1994). After recording basal pancre-
atic nerve discharges for 5 min, STG was infused at doses of 6.0 and 60.0 μg, i.c.v. Pancreatic nerve discharges were monitored for 30 min. Immediately before sacrifice, methylene blue (2 μL, i.c.v.) was administered to verify the injection site. Data from animals that did not display the dye throughout their ventricular system were excluded from the analyses.

**CHRONIC DECELERATION.** We have shown that chronic deceleration decreases basal pancreatic enzyme secretion and reduces the net increase in pancreatic secretion stimulated by intraduodenal infusion of peptone (Li et al. 2003). To quantify hypoglycemia-induced pancreatic nerve responses contributed by the rat forebrain, we performed chronic deceleration, which is a classical method designed to remove the influence of the forebrain. The rat is placed in a stereotaxic instrument, the dura is removed, and the brain is transected at the supracollicular level in a 2-stage procedure using a handheld spatula (DiRocco and Grill 1979). Wounds are closed with sterile sutures and wound margins are treated with topical and systemic antibiotics (Kellin, 25 mg kg⁻¹, subcutaneous). Completeness of each transection was verified histologically. Pancreatic nerve recording studies were performed 7 to 10 days after deceleration.

**NURSING CARE.** Chronically decerebrate rats exhibit relative immobility; however, they are sufficiently coordinated to groom their fur and they often overreact with well-coordinated movements such as running and jumping (Tang 1955). Although incapable of effective thermoregulation or spontaneous feeding or drinking, they are able to consume orally administered fluids (Steiner 1973).

Body temperature was maintained between 34 and 37.5°C by warming or evaporative cooling. Rectal temperature was recorded 3 times daily. The decerebrate rats and the controls were tube-fed three 12-ml meals daily consisting of equal parts sweetened condensed milk and water (with a multiple vitamin supplement). The animals were housed individually and subjected to a 12-h day/12-h night cycle.

**BILATERAL CHEMICAL LESIONS IN THE LATERAL HYPOTHALAMIC AREA OR ARCUATE NUCLEUS.** To identify the brain sites responsible for modulating vagal efferent firing evoked by insulin-induced hypoglycemia, LHA or arcuate nucleus (ARC) lesion studies were performed. The anesthetized rats were placed in a stereotaxic instrument with the skull oriented horizontally between the bregma and lambda. A midline incision was made in the scalp to expose the lambda and bregma. A micropipette (OD of the tip, 40–60 μm) was stereotactically lowered into the LHA, 2.0 mm posterior to the bregma, 1.9 mm on each side of the midline, and 7.8 mm below the skull surface. Kainic acid (4.8 mmol/l, 60 nl) in sterile phosphate-buffered saline (PBS) was administered using a microinjector. ARC lesions were made in a separate group of rats at the following coordinates: 3.0 mm posterior, 0.4 mm lateral, 10.0 mm deep. Sham lesions were made overnight at room temperature in blocking solution, PBS–Triton X-100 containing mouse monoclonal NPY antibodies (1:400) (Grouzmann et al. 1992). The sections were then rinsed in PBS, incubated for 30 min at 37°C with FITC-conjugated donkey antimouse antibody (1:40, Jackson ImmunoResearch Laboratories, West Grove, PA), rinsed in PBS again, and mounted in a mixture of glycerol and PBS (3:1) containing 0.1% p-phenylenediamine (Sigma-Aldrich).

Specificity of the immunostaining patterns was demonstrated by the degree of preabsorption of the antiserum with its corresponding antigen at a concentration of 10⁻⁶ mol/l. Sections were examined using a Chroma optical filter set (Chroma Technology, Rockingham, VT), and images were processed on the computer attached to the scope using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA). The ARC was divided into 3 levels according to the atlas reported by Paxinos and Watson (1998): rostral (bregma: about −2.12 to −2.30 mm), mid (bregma: −2.80 mm), and caudal (bregma: −3.80 mm). Neurons were counted in 2 sections from each level per animal (n = 4). Only neuron profiles displaying a cell nucleus were counted.

**Microinjection of orexin-A receptor antagonist SB-334867 or NPY receptor antagonist [d-Trp²⁴⁶-d-Thr²³]NPY₂⁷–₃₆ DMV CANNULATION.** Brain nuclei microinjection during electrophysiological recording of the vagal pancreatic efferent nerve was achieved using an adaptation of a technique described by Micheliné and Bonagamba (1988), which facilitates the implantation of bilateral guide cannulas in the direction of the DMV. A 15-mm long stainless steel cannula (24 gauge) was introduced perpendicularly through a small opening, 14 mm caudal to the bregma, 0.5 mm lateral to the midline, and 8.3 mm below the skull surface. The guide cannula was fixed to the skull with methacrylate and wash screws and closed with an occluder until the experiments were begun. Microinjection into the DMV was accomplished using a 33-gauge needle, 1.5 mm longer than the guide cannula and attached with polyethylene tubing to a pneumatic PicoPump (model PV830, World Precision Instruments, Sarasota, FL). Microinjections were delivered in volumes of 20 to 35 nl for 15 s. The placement of the needle tip in the DMV was subsequently confirmed histologically. Wounds were closed with sterile sutures and wound margins were treated with topical and systemic antibiotics (Kellin, 25 mg kg⁻¹, subcutaneous). After DMV cannulation, each rat was given diluted Tylenol (50 mg kg⁻¹) orally for 24 h to reduce postoperative pain. Chronic DMV cannulation did not appear to cause distress. Behavior and feeding habits were normal; in fact the rats gained an average of 6.1 g in 7 days. After a 4-day recovery period, the rats were reanesthetized and pancreatic nerve recordings were performed. At the end of the study, the brain was removed and histological sections were examined microscopically. The microinjection sites, which were determined to be the point of termination of the cannula track, were marked on plates reproduced from the atlas of Paxinos and Watson (1998).

Using a similar cannula injection technique, previous studies have shown that after microinjection of [³H][3-methyl-His]-TRH (50 nl) into the preoptic nucleus, more than 75% of the radioactivity is found within a diameter of 600 μm from the injection site (Siren et al. 1991). Further, this same technique recently showed that microinjection of ACh into the lateral hypothalamic nucleus or the paraventricular nucleus induced an increase in pancreatic protein output; however, microinjection of ACh into the DMV had no effect on pancreatic secretion (Li et al. 2003). The involvement of CNS orexin and NPY in the mediation of vagal pancre-
HYPOGLYCEMIA STIMULATES VAGAL PANCREATIC EFFERENT

Effect of insulin-induced hypoglycemia on vagal pancreatic efferent nerve activity

In the basal state, blood glucose measured 114 ± 5 mg dl\(^{-1}\). During insulin infusion at the constant rate of 2.0 mU kg\(^{-1}\) min\(^{-1}\), steady-state blood glucose levels were measured. Plateaus at blood glucose levels of 86, 74, and 57 mg dl\(^{-1}\) were targeted for the concentration response experiments (Fig. 1A). The amount of glucose needed to maintain the predetermined levels of glycemia for the course of the experiments is shown in Fig. 1B. A decreasing glucose infusion rate corresponds with a lower level of glycemia. The total amount of glucose infused was the same for all experiments, whether the study involved intact rats, chronically decerebrate rats, or rats after vagal afferent rootlet section plus splanchnicotomy.

Data were collected from 58 unitary recordings of the pancreatic vagal efferent nerves of 20 rats. Before insulin infusion, all units displayed very low spontaneous activity (0 to 2.5 impulses 30 s\(^{-1}\)). A total of 43 of 58 units responded to insulin-induced hypoglycemia. Pancreatic nerve discharges did not significantly increase at blood glucose levels above 90 mg dl\(^{-1}\). The frequency of vagal efferent discharges increased in response to the falling blood glucose levels. The threshold was measured at 86 ± 4 mg dl\(^{-1}\); the discharge frequency increased from a basal rate of 1.1 ± 0.3 to 4.0 ± 1.1 impulses 30 s\(^{-1}\). The maximal response—an increase in the pancreatic efferent nerve firings to 19 ± 3 impulses 30 s\(^{-1}\)—was evoked at a blood glucose level of 74 ± 6 mg dl\(^{-1}\). Original action potential recordings are presented in Fig. 2, A and B. The minimum increase in discharge frequency, from a basal rate of 1.1 ± 0.3 to 1.8 ± 1.0 impulses 30 s\(^{-1}\), was observed at a blood glucose level about 57 ± 5 mg dl\(^{-1}\). Discharge frequency data of the pancreatic efferent nerve in response to insulin-induced hypoglycemia are presented in Fig. 3.

Effect of vagal afferent rootlet section plus splanchnicotomy on vagal pancreatic efferent nerve activity stimulated by insulin-induced hypoglycemia

Data were collected from 7 rats after vagal afferent rootlet section plus splanchnicotomy. After surgical denervation, hypoglycemia (i.e., blood glucose level, 69–80 mg dl\(^{-1}\)) evoked an increase in pancreatic efferent nerve discharges from 1.0 ± 0.25 to 18.2 ± 4 impulses 30 s\(^{-1}\) in 22 units tested. Figure 2C shows one original action potential recording. Thus compared with rats with intact vagal and splanchnic nerves, extrinsic afferent nerve denervation appeared to have no effect on pancreatic nerve firing in response to insulin-induced hypoglycemia.
Effects of decerebration on vagal pancreatic efferent nerve activity stimulated by insulin-induced hypoglycemia

Data were collected from 38 unitary pancreatic nerve recordings in 4 sham-operated and 9 decerebrate rats. Both sham-operated and decerebrate rats gained weight: from 227 ± 7 and 230 ± 4 g to 260 ± 7 and 258 ± 10 g, respectively, on the 10th postoperative day. Chronic decerebration did not change the blood glucose level or the basal firing rate of the pancreatic efferent nerve. Insulin-induced hypoglycemia, however, evoked an increase in the pancreatic nerve discharge frequency in decerebrate rats: 1.4 ± 0.2 to 8.0 ± 0.9 impulses 30 s⁻¹ at blood glucose levels of 70 to 80 mg dl⁻¹. Compared
with the sham operation, chronic decerebration inhibited pancreatic nerve firings in response to insulin-induced hypoglycemia by 56% (Fig. 5, A and B and Fig. 6A). These observations suggest that the forebrain is required for the full expression of pancreatic nerve responses stimulated by hypoglycemia.

Effects of bilateral lesions of the LHA or ARC on vagal pancreatic efferent nerve activity stimulated by insulin-induced hypoglycemia

Kainic acid was injected into the LHA (n = 6) and ARC (n = 6). Successful bilateral lesions of the LHA were confirmed histologically in 5 rats (Fig. 7). The 6th rat exhibited incidental bilateral damage in the zona incerta, in addition to LHA damage. The vagal nerve responses of this rat were eliminated during insulin-induced hypoglycemia; data were not included in the analysis. Successful bilateral ARC lesions were confirmed in 4 of 6 rats. Cresyl violet staining showed destruction of most cell bodies within the ARC (Fig. 8B). Of the 2 remaining rats, lesioning was incomplete in one and ventromedial hypothalamus damage occurred in the other. Vagal nerve firing in response to hypoglycemia in these 2 rats was not significantly different from that in rats with sham lesions.

Immunohistochemical studies confirmed NPY-immunoreactivity (NPY-ir) in small-diameter cell bodies clustered in the ventromedial ARC, sometimes extending into the median eminence (Fig. 9A). NPY-ir was greatly diminished by kainic acid lesioning (Fig. 9B).

Thus data were collected from 28 unitary pancreatic nerve recordings of 5 rats with LHA lesions and 4 rats with ARC lesions. At the blood glucose level of 76 ± 4 mg dl⁻¹, pancreatic nerve discharge rates in rats with ARC or LHA lesions were 19 ± 6 and 8.9 ± 2 impulses 30 s⁻¹, respectively. Original action potential recordings are shown in Fig. 5, C and D. Discharge frequency data of the vagal pancreatic efferent nerve in response to insulin-induced hypoglycemia are presented in Fig. 6A. A separate group of rats underwent sham lesions, which involved injections of saline into the LH (n = 4) or ARC (n = 4). Increased vagal pancreatic nerve firing was observed during hypoglycemia (Fig. 6A). Compared with nonoperated rats, pancreatic nerve firing during hypoglycemia in the sham-lesioned rats did not change significantly.

Effects of i.c.v. administration of 5TG or insulin on vagal pancreatic efferent nerve activity

Data were collected from 3 sham-operated rats and 4 rats with LHA lesions. In the sham-operated rats, an i.c.v. injection of 6.0 µg 5TG had no effect on vagal pancreatic efferent nerve discharges, whereas a 60.0-µg dose increased pancreatic nerve firing by 10.220.33.6 on August 14, 2017 http://jn.physiology.org/ Downloaded from

FIG. 4. Response of the vagal nodose ganglia neurons to insulin-induced hypoglycemia. A: vagal nodose ganglia neurons failed to respond to insulin-induced hypoglycemia (BG level, 76 mg dl⁻¹). B: in contrast, intraduodenal perfusion of serotonin [5-hydroxytryptamine (5-HT)] during insulin-induced hypoglycemia stimulated nodose neuronal firing.

FIG. 5. Effect of decerebration and of bilateral chemical lesions of the arcuate nucleus or lateral hypothalamic area on the response of the vagal pancreatic nerve to insulin-induced hypoglycemia. A: in sham-operated rats, insulin-induced hypoglycemia produced a marked increase in pancreatic nerve firing, similar to the increase observed in nonoperated rats (see Fig. 2B). B: chronic decerebration inhibited pancreatic nerve discharge evoked by hypoglycemia by 56%. C: arcuate nucleus (ARC) lesions had no effect on hypoglycemia-induced pancreatic nerve firing. D: lateral hypothalamic area (LHA) lesions, however, reduced pancreatic nerve firing by 51%. BG, blood glucose.
from 0.5 \pm 0.5 to 21 \pm 3.5 impulses 30 s\(^{-1}\). Figure 10A shows one original action potential recording. These observations confirm that, similar to systemic hypoglycemia, central hypoglycemia stimulates vagal efferent activity. Bilateral LHA lesions markedly inhibited vagal pancreatic efferent nerve discharges stimulated by i.c.v. 5TG (Fig. 10B), which suggests that LHA neurons mediate vagal pancreatic nerve firing stimulated by central hypoglycemia. In contrast, i.c.v. bolus injections of insulin at doses of 5.0 and 20.0 mU had no effect on pancreatic efferent nerve firing, which suggests that the increase in pancreatic nerve activity in response to intravenous infusion of insulin at a dose of 2.0 mU kg\(^{-1}\) min\(^{-1}\) was not caused by the direct action of insulin on the CNS. Discharge frequency data of the pancreatic efferent nerve are presented in Fig. 6B.

**FIG. 6.** Discharge of the vagal pancreatic efferent nerve in response to insulin-induced hypoglycemia and to intracerebroventricular 5-thioglucose (5TG). A: effects of decerebration and of bilateral chemical lesions of the ARC and the LHA. Data were collected from 38 unitary recordings in 4 sham-operated and 9 decerebrate rats. Chronic decerebration did not change basal pancreatic efferent nerve firing. Compared with sham operation, decerebration inhibited pancreatic nerve discharges elicited by hypoglycemia by 56%. In separate studies, data were collected from 31 unitary recordings in 4 rats with ARC lesions and 5 rats with LHA lesions. Compared with sham lesions, LHA lesions inhibited pancreatic nerve firing by 54%. No difference was observed with ARC lesions. All values are means \pm SE. *P < 0.01 compared with sham operation or sham lesion. B: discharge of the vagal pancreatic efferent nerve in response to intracerebroventricular (i.c.v.) infusion of insulin or 5TG, and in response to microinjection of orexin into the DMV. Data were collected from 3 sham-lesioned rats, 4 rats with LHA lesions, and from 5 rats after microinjection of orexin into the DMV. All values are means \pm SE. *P < 0.01, compared with spontaneous firing. **P < 0.01, compared with sham lesions. BG, blood glucose.

**FIG. 7.** Chemical lesions of the lateral hypothalamic area. Representative coronal sections (bregma, -1.80 mm) stained with cresyl violet from rats after microinjection of PBS (A) or kainic acid (B). Most cell bodies within the LHA were destroyed by kainic acid. C and D: higher magnifications of the insert in A and B, respectively. PVN, paraventricular nucleus; 3V, 3rd ventricle.
Effects of microinjection of orexin-A or NPY receptor antagonists into the DMV on pancreatic nerve activity stimulated by insulin-induced hypoglycemia

The left cervical vagus was carefully separated from the carotid artery and then sectioned. DMV microinjection (right side) studies were performed to identify the neurotransmitters responsible for mediating pancreatic efferent nerve activity during hypoglycemia. Microinjections of orexin-A (n = 8) or NPY receptor antagonists (n = 8) were administered. The injection sites were located outside of DMV in 2 rats that received the orexin antagonist and in 3 rats that received the NPY receptor antagonist (Fig. 11A). Compared with vehicle injection in the DMV, microinjection of the NPY receptor or orexin antagonists outside the DMV did not change the pancreatic nerve firings induced by hypoglycemia. Data were collected from 6 rats that received the orexin-A receptor antagonist and 5 rats that received the NPY receptor antagonist. Microinjection of the D-NPY27–36 at a dose of 5.0 μg into the DMV had no effect on pancreatic vagal efferent nerve discharge stimulated by insulin-induced hypoglycemia. One original action potential recording is presented in Fig. 12B. Previous studies have shown that this treatment suppresses pancreatic vagal efferent firing evoked by intraluminal stimuli (Wu et al. 2001, 2002). In contrast, microinjection of the orexin-A receptor antagonist SB-334867 at a dose of 5.0 ng into the DMV inhibited pancreatic nerve discharge in response to insulin-induced hypoglycemia by 53% (Fig. 12A). Discharge frequency data of the pancreatic efferent nerves are presented in Fig. 3.

Effects of microinjection of orexin into the DMV on pancreatic vagal efferent nerve activity and blood glucose level

The locations of orexin microinjections into the dorsal vagal complex are shown in Fig. 11B. The injection sites were within the DMV in 5 of 8 rats studied. Data were collected from 16 unitary recordings of those 5 rats. Unilateral microinjection of orexin at a dose of 20.0 pmol evoked a marked increase in pancreatic vagal efferent nerve firings, from a basal level of 1.5 ± 3 to 29 ± 5 impulses 30 min⁻¹. The peak lasted for 3 min and activity gradually returned to basal 40 min after the injection. One original action potential recording is shown in Fig. 12C. At 10 and 20 min after microinjection of orexin into the DMV, blood glucose levels increased from a basal level of 116 ± 8 mg dl⁻¹ to 225 ± 5 and 251 ± 7 mg dl⁻¹, respectively.

DISCUSSION

Hypoglycemia, which often results from intensive insulin therapy, is a major complication of type 1 (insulin-dependent) diabetes mellitus and causes recurrent physical and psychological morbidity. Also associated with significant mortality rates, hypoglycemia remains a major limiting factor in improving glycemic control (Frier and Fisher 1999). Blood glucose concentration is maintained largely by the release of insulin, glucagon, and catecholamine from the pancreas and adrenal gland (Cryer 1993; Taborsky 2001). The accepted view is that low circulating glucose levels act directly on the pancreatic islets to induce insulin and glucagon release. The autonomic nervous system is known to be important in controlling the counterregulatory response to insulin-induced hypoglycemia (Coiro et al. 1989; Dunning and Taborsky 1991; Strubbe and Steffens 1993; Taborsky 2001). Hypoglycemia activates 3 autonomic inputs to the pancreas: the pancreatic parasympathetic nerve (Havel et al. 1992; Schwartz et al. 1978), the sympathetic nerve (Strubbe and Steffens 1993), and the release of epinephrine from the adrenal medulla (Cannon et al. 1924). The mechanisms responsible for hypoglycemia-induced vagal and sympathetic efferent signaling to the pancreas have been largely unexplored.

FIG. 8. Chemical lesions of the arcuate nucleus. A: coronal section (bregma, −3.30 mm) from a rat that received an injection of PBS into the arcute nucleus (Arc). B: a well-placed injection of kainic acid caused bilateral damage. Sections were stained with cresyl violet.

FIG. 9. Effect of the neurotoxin kainic acid on NPY-immunopositive neurons in the arcuate nucleus. A: NPY-immunopositive neurons in a section of the arcuate nucleus (bregma, −3.30 mm) from control rat with a sham lesion. B: few NPY-positive neurons are visible in a section of the arcuate nucleus after injection of kainic acid.
Niijima (1975) reported that hypoglycemia decreased pancreatic vagal nerve activity in rabbits. The degree of hypoglycemia may be one of several critical factors that influence vagal pancreatic nerve responses. Niijima observed that intravenous administration of insulin at 20.0 U kg$^{-1}$ evoked a decrease in pancreatic nerve firing when blood glucose levels dropped to 20 mg dl$^{-1}$. This result contradicted a great body of evidence that suggested that insulin-induced hypoglycemia stimulated pancreatic nerve responses. A: 5TG infusion (60 μg, i.c.v.) markedly stimulated vagal pancreatic nerve firing. B: bilateral ablation of LHA tissue suppressed this response.

**FIG. 10.** Response of the pancreatic efferent nerve to intracerebroventricular infusion of 5TG. A: 5TG infusion (60 μg, i.c.v.) markedly stimulated vagal pancreatic nerve firing. B: bilateral ablation of LHA tissue suppressed this response.

**FIG. 11.** Location of microinjection sites in the dorsal vagal complex. Drawings of coronal sections adapted from the atlas of Paxinos and Watson (1998) show the microinjection sites in the dorsal vagal complex. Numbers at the upper right of each section indicate the distance from the bregma. A: two separate groups of rats were used in these studies. Rats in group I received right-sided DMV injections of the vehicle and orexin-A receptor antagonist SB-334867. The injection sites are shown on the left side of dorsal vagal complex. Rats in group II received right-sided DMV injections of vehicle or NPY receptor antagonist (n-Tyr$^{27,36}$, n-Thr$^{27}$)NPY$^{27-36}$. Injection sites are labeled on the right side of the dorsal vagal complex. No rat received more than 2 DMV injections. Eight rats were tested in each group. Histological sections showed that the point of termination of the injection-needle track was within the DMV in 6 rats in group I and 5 rats in group II. Black circles show the sites within the DMV. Black squares show the injection sites outside the DMV (in the NTS or hypoglossal nucleus). B: locations of microinjection of orexin in the dorsal vagal complex. Eight rats were used in this study. In 5 rats, the injection sites were within the DMV and are shown at the right side of the dorsal vagal complex (black circles). In 3 rats, the injection sites were outside the DMV (near the border of the hypoglossal nucleus for 2 rats, and in the NTS for 1 rat). These sites are shown on the left side of the dorsal vagal complex (black squares). No change in pancreatic nerve firing stimulated by insulin-induced hypoglycemia was observed in these 3 rats. Only data from rats with appropriately placed microinjection sites were analyzed. AP, area postrema; CC, central canal; DMV, dorsal motor nucleus of the vagus; NTS, nucleus tractus solitarius; 12, hypoglossal nucleus; 4V, 4th ventricle.
gastric acid, bile, and pancreatic enzyme secretions and increased plasma pancreatic polypeptide concentration, a surrogate marker of pancreatic vagal tone. Researchers have traditionally used insulin-induced hypoglycemia to stimulate vagal efferent activity. In our study, electrophysiological recordings of the vagal pancreatic efferent nerve were made during stepped hypoglycemic clamp experiments. Pancreatic nerve discharges did not significantly increase at blood glucose levels above 90 mg \(\text{dl}^{-1}\). The frequency of efferent discharge increased in response to the falling blood glucose levels. The threshold was measured at 86 ± 4 mg \(\text{dl}^{-1}\). The maximal response—an increase in pancreatic efferent nerve firing from a basal rate of 1.1 ± 0.3 to 19 ± 3 impulses 30 s\(^{-1}\)—was evoked at a blood glucose level of 74 ± 6 mg \(\text{dl}^{-1}\). In our anesthetized rat studies, minimal increases in the discharge frequency of pancreatic vagal efferent nerves were observed at blood glucose levels about 57 ± 5 mg \(\text{dl}^{-1}\). Plasma glucose is regulated at higher levels in rats than in humans; however, it is noteworthy that, in humans, pancreatic polypeptide levels increase even when the blood glucose concentration falls to 40 mg \(\text{dl}^{-1}\). Species differences and the effects of the anesthetics used in current studies may explain the less-sensitive vagal pancreatic efferent nerve responses during severe hypoglycemia observed in anesthetized rats. Previous studies have shown that anesthesia induced by pentobarbital attenuates the epinephrine response to hypoglycemia, and low-dose halothane is minimally suppressive (Havel et al. 1992). Ketamine was used in this study because its effects on glycemia are minor (Aynsley-Green et al. 1973). Severe metabolic stress-related neurotransmitters and CNS damage in anesthetized rats may also suppress pancreatic efferent neural activities.

Increased vagal pancreatic efferent nerve activity during insulin-induced hypoglycemia has important physiological significances. Previous studies have shown that electrical stimulation of the vagus nerve, in addition to increasing glucagon secretion, which mimics the response during hypoglycemia, also increases insulin secretion. However, because vagal effects on islet hormone secretion are critically dependent on the ambient glucose level, hypoglycemia should markedly potentiate the glucagon response and attenuate the insulin response. Thus vagal activation during hypoglycemia may contribute to the stimulation of glucagon secretion without significantly stimulating insulin secretion (Taborsky 2001). Interestingly, vagal inhibition of insulin secretion has been observed under fasting conditions (Blat and Malpert 2001).

Systemic hypoglycemia may activate peripheral glucose sensors located in the liver, the portal vein, and the neurons of enteric nervous system (Hevener et al. 2001; Jackson et al. 2000; Liu et al. 1999). Afferent innervation of the portal vein is important for mediating sympathoadrenal responses induced by hypoglycemia (Hevener et al. 2000). To ascertain whether hypoglycemia activates vagal primary afferent neurons, we recorded the electrophysiological activity of the nodose ganglia neurons. We observed that all recorded units of the nodose ganglia were either silent or displayed very low spontaneous activity (0 to 3 impulses min\(^{-1}\)). Insulin-induced hypoglycemia did not alter vagal nodose neuronal firing. On the other hand, the control experiment showed that intraduodenal perfusion of 5-HT solution during hypoglycemia stimulates nodose neuronal firings. Our previous research showed that carbohydrates in the intestinal lumen activate enterochromaffin cells to release 5-HT (Li et al. 2001). Intraduodenal perfusion of maltose and glucose stimulates vagal node neurons by the release of endogenous 5-HT from the mucosal enterochromaffin cells, which acts on the 5-HT\(_3\) receptors on vagal afferent fibers (Zhu...
et al. 2001). Intraluminal perfusion of 5-HT stimulates vagal nodose neurons and increases pancreatic secretion.

Neurons in the brain directly sense changes in glucose levels, although whether the CNS can actually detect small changes in blood glucose and in turn initiate pancreatic nerve activation remain uncertain. To identify the sites of action at which hypoglycemia stimulates pancreatic nerve firing, we performed bilateral vagal afferent rootlet section plus splanchicotomy to completely exclude the transmission of peripheral afferent signals to the CNS during stepped hypoglycemic clamp experiments. Our results showed that surgical denervation had no effect on the vagal pancreatic nerve firing stimulated by hypoglycemia, suggesting a central site of action. Our results are compatible with the Fos expression in the brain nuclei induced by hypoglycemia in the rats after acute cervical vagotomy (Yuan and Yang 2002). These observations indicate that, unlike postprandial pancreatic enzyme secretion, which is mainly mediated by the vago-vagal reflex, vagal pancreatic efferent nerve responses to insulin-induced hypoglycemia are unaffected by the vago-vagal reflex (Li et al. 2000, 2001). Our studies indicate that hypoglycemia primarily activates glucose-sensitive neurons in the brain, which in turn stimulate pancreatic nerve firing; peripheral signals do not play a critical role in mediating these responses. Our findings also corroborate observations made after surgical hepatic denervation and vagal cooling in dogs—the normal counterregulatory response to insulin-induced hypoglycemia does not require afferent signaling from the liver (Jackson et al. 2000) or by the vagal nerves (Cardin et al. 2001).

Under physiological conditions the brain is fueled almost exclusively by glucose. Unable to synthesize or store glucose, the brain depends on the maintenance of an adequate blood glucose concentration to function (Clarke and Sokoloff 1999). At normal blood glucose levels, the rate of blood–brain glucose transport is about twice that of brain glucose metabolism (Blomqvist et al. 1991). However, as the arterial glucose concentration falls below the physiological range, blood–brain glucose transport decreases to the point that it limits brain glucose metabolism. In the anesthetized rat brain, extracellular glucose levels increase in hyperglycemia and decrease in hypoglycemia, paralleling the changes in blood glucose (Silver and Ereenska 1994). The blood glucose level of normoglycemic rats was 7.6 mmol/l, compared with 2.4 mmol/l in the brain. Insulin-induced hypoglycemia lowered the blood glucose level to 2.8 mmol/l and the brain glucose level to 0.16 mmol/l. Cerebral dysfunction generally develops at blood glucose levels about 3 mmol/l (Heller and Macdonald 1996). Strachan and colleagues (2001) reported that acute hypoglycemia impaired human CNS function. Acute hypoglycemia evoked changes in the electroencephalogram (Pramling et al. 1988) and in the brain stem evoked potential (Jones et al. 1990). Significant impairment of cognitive functions was observed when blood glucose concentration dropped from basal level 5.0 to 2.6 mmol/l. Previous studies have shown impairment of human cerebral function at a blood glucose level of 54 mg dl⁻¹ (Heller and Macdonald 1996). The compound 5TG inhibits glucose utilization and causes intracellular hypoglycemia. Not surprisingly, we observed that i.c.v. 5TG induced a marked increase in pancreatic nerve firing.

Neurons in the brain can directly sense changes in glucose levels. Glucose-sensing neural elements have been identified in the LHA, the ventral medial hypothalamus (VMH), the nucleus of the solitary tract (NST), the LHA, the ventral medial hypothalamus (VMH), the nucleus of the solitary tract (NST), the DMV, ventral lateral medulla, and preganglionic spinal cord neurons, which all project to the pancreas (Buijs et al. 2001). Thirty percent of orexin-immunoreactive neurons were shown...
to display Fos-like immunoreactivity during insulin-induced hypoglycemia (Cai et al. 2001). Furthermore, hypothalamic prepro-orexin mRNA levels are increased after 48 h of fasting and during acute insulin-induced hypoglycemia (Sakurai et al. 1998), which suggests that these neurons are activated under conditions of hunger. Our study showed that microinjection of an orexin-A receptor antagonist inhibited pancreatic nerve discharge by 53%. In contrast, microinjection of orexin into the DMV stimulated pancreatic efferent nerve firing and increased blood glucose levels. These observations support the hypothesis that central glucoprivation activates subpopulations of LHA neurons containing orexin. The released orexin acts on DMV neurons, which, in turn, stimulate pancreatic vagal pathways.

Another neuropeptide that may play a role in the descending hypothalamic–brain stem pathway is NPY. Previous studies have shown that i.c.v. administration of NPY increases gastric, pancreatic, and biliary secretions (Farouk et al. 1992; Geoghegan et al. 1993). We have shown that central administration of NPY stimulates pancreatic nerve firing and increases enzyme secretion in conscious rats (Wu et al. 2001). Central administration of 2-deoxy-D-glucose has been shown to induce c-fos gene expression in NPY neurons (Minami et al. 1995). Glucoprivation has been shown to induce an increase in NPY mRNA expression in the ARC (Fraly and Ritter 2003). However, we found that bilateral ARC lesions did not significantly affect pancreatic nerve firing evoked by insulin-induced hypoglycemia. Microinjection of an NPY antagonist had no effect on pancreatic nerve firing stimulated by insulin-induced hypoglycemia. Immunohistochemistry studies have shown that the LHA receives a dense innervation of NPY-ir nerve terminals. Further, with double staining, NPY-ir terminals can be seen around and sometimes in close relation to orexin-ir cell bodies in the LHA (Broberger et al. 1998). Therefore the influence of NPY on LHA orexin-ir neurons in the modulation of vagal efferent inputs to the pancreas during hypoglycemia cannot be ruled out.

Insulin receptors exist within the hypothalamic areas (Unger et al. 1986), and plasma insulin can reach the brain by an insulin receptor–mediated system in the cerebrospinal fluid (Baura et al. 1993). In this study, we showed that i.c.v. injection of insulin at doses of 20.0 mU/kg does not affect vagal pancreatic efferent nerve firing, which suggests that the increase of pancreatic nerve firing stimulated by intravenous perfusion of insulin at a dose of 2.0 mU kg⁻¹ min⁻¹ was not caused by the direct action of insulin on the brain.

In summary, we have demonstrated that systemic hypoglycemia stimulates vagal pancreatic nerve firing through a central mechanism. The brain stem contains the neural network to mediate pancreatic nerve activities stimulated by hypoglycemia. However, the forebrain also plays an important role in enhancing these responses. Central glucoprivation activates subpopulations of LHA neurons containing orexin. The released orexin acts on preganglionic DMV neurons to stimulate pancreatic efferent nerve firings and thus regulate pancreatic functions.

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