Rostroventrolateral medullary neurons modulate glucose homeostasis in the rat

A. J. M. Verberne and D. M. Sartor

Clinical Pharmacology and Therapeutics Unit, Department of Medicine, Austin Health, University of Melbourne, Heidelberg, Victoria, Australia

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Verberne AJ, Sartor DM. Rostroventrolateral medullary neurons modulate glucose homeostasis in the rat. Am J Physiol Endocrinol Metab 299: E802–E807, 2010. First published August 31, 2010; doi:10.1152/ajpendo.00466.2010.—Several lines of evidence support the view that the premotor sympathetic input to the adrenal gland arises from the rostroventrolateral medulla (RVLM). The aim of this study was to determine whether RVLM neurons play a role in glucose homeostasis. We identified RVLM neurons that control epinephrine secretion by searching for medullospinal neurons that responded to neuroglucoprivation induced by systemic 2-deoxyglucose (2-DG) administration. We tested the effect of disinhibition of the RVLM on arterial blood pressure and plasma glucose concentration. RVLM medullospinal barosensitive neurons (n = 17) were either unaffected or slightly inhibited by 2-DG. In contrast, we found a group (n = 6) of spinally projecting neurons that were excited by 2-DG administration. These neurons were not barosensitive and had spinal conduction velocities in the unmyelinated range (<1 m/s). These neurons may mediate epinephrine secretion and participate in the counterregulatory responses to neuroglucoprivation. To test the hypothesis that activation of the RVLM leads to adrenomedullary activation and subsequent hyperglycemia, we applied the GABA<sub>A</sub> antagonist bicuculline to the RVLM and measured blood pressure, heart rate, and blood glucose in rats with intact adrenals or after bilateral adrenalectomy. Disinhibition of the RVLM resulted in hypertension, tachycardia, and hyperglycemia (4.9 ± 0.3 to 14.7 ± 0.9 mM, n = 5, P < 0.05). Adrenalectomy significantly reduced the hyperglycemic response but did not alter the cardiovascular responses. These data suggest that the RVLM is a key component of the neurocircuitry that is recruited in the counterregulatory response to hypoglycemia.

glucose homeostasis; counterregulation; sympathetic nervous system; adrenal glands

EPINEPHRINE IS A GLUCOSE-COUNTERREGULATORY HORMONE that is of major importance in countering hypoglycemia in individuals with type 1 and advanced type 2 diabetes (7). Epinephrine is synthesized by chromaffin cells in the adrenal medulla and its secretion is controlled by a distinct group of sympathetic preganglionic neurons (21). It has long been assumed that epinephrine is an important modulator of cardiovascular function, but it probably contributes minimally to blood pressure regulation (38). Indeed, it is likely that its primary role is as a metabolic hormone which serves to mobilise glucose by activation of liver glycogenolysis and gluconeogenesis (13).

In contrast to the sympathetic preganglionic neurons that control vasomotor tone, the sympathetic preganglionic neurons that control epinephrine secretion are not particularly responsive to baroreceptor stimulation. Instead, they are strongly activated by neuroglucoprivation induced by administration of the glucoprivic agent 2-deoxyglucose (2-DG) (21), a glucose analog that does not undergo glycolysis. Insulin-induced hypoglycemia and 2-DG-induced neuroglucoprivation activate several groups of neurons (glucose-sensing neurons) that are located in the medulla and the hypothalamus (15, 24, 26, 28). This results in adrenal catecholamine and glucagon secretion accompanied by inhibition of insulin secretion (2). There is evidence that adrenomedullary sympathetic preganglionic neurons receive premotor input from the neurons in the rostroventrolateral medulla (RVLM) (20, 21), a brain region that is critically involved in regulation of sympathetic vasomotor outflow (11). However, the properties of the neurons responsible for control of adrenomedullary secretion have not been identified.

The aims of the present study were threefold. We sought 1) to identify spinally projecting neurons in the RVLM that were activated by neuroglucoprivation induced by systemic administration of 2-DG, 2) to determine whether the properties of these neurons are different from the properties of RVLM presympathetic vasomotor neurons, and 3) to determine whether excitation of these neurons leads to hyperglycemia.

METHODS

Animals. All experiments were performed using male Sprague-Dawley rats (300–420 g, n = 33) obtained from the Animal Resources Centre, Perth, Western Australia. This study was approved by the Animal Ethics Committee of Austin Health (Heidelberg, Victoria, Australia) and complied with principles outlined in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Surgical preparation. Rats were initially anaesthetized with isoflurane. A tracheostomy was then performed, and artificial respiration (50–60 breaths/min; 10 ml/kg) was commenced (100% O<sub>2</sub>, 1.5–1.7% isoflurane). Arterial and venous cannulation for measurement of arterial blood pressure and for intravenous drug administration, respectively, were carried out as described previously (31, 36). After completion of the cannulations, the abdominal artery was exposed near the crus of the diaphragm through a midline abdominal incision. An inflatable cuff was then placed on the abdominal aorta. Inflation of the cuff led to a graded increase in arterial blood pressure, which was used to activate arterial baroreceptors. Two separate sets of experiments were performed. First, by use of extracellular single-unit recording techniques, the RVLM was explored for spontaneously active neurons that had axonal projections to the thoracic spinal cord. Second, the RVLM was stimulated using bilateral local microinjections of the GABA<sub>A</sub> receptor antagonist bicuculline methiodide.

Extracellular single-unit recording. Extracellular single-unit recording of RVLM presympathetic neurons (5, 31, 36) was performed using glass microelectrodes (2 mm OD) filled with 2% Pontamine sky...
blue in 0.5 M sodium acetate via a transcerebellar approach. A bandpass amplifier (400 Hz to 4 kHz) and window discriminator (Fintronics, Orange, CT) was used to record extracellular action potentials, which were monitored using an oscilloscope and an audio amplifier. The procedures used for location, identification, and extracellular single-unit recording of RVLM neurons have been described previously (5, 31, 36). Briefly, the location of the RVLM was identified by recording antidromic field potentials in the VLM elicited by electrical stimulation (0.5 Hz, 0.1 ms, 0.3–1.0 mA) of the mandibular branch of the facial nerve. The initial penetration of the brain with the microelectrode was usually made 3.0 mm caudal to the lambdoidal suture and 2.0 mm lateral to the midline. A bipolar electrode was placed into the dorsolateral funiculus of the spinal cord for antidromic activation of RVLM neurons, while another bipolar electrode was placed on the mandibular branch of the facial nerve. Electrical stimulation applied to this electrode evoked field potentials that were used to identify the caudal boundaries of the facial motor nucleus and subsequently the location of RVLM neurons. We searched for spontaneously active units in the region of the medulla within 500 μm of the caudal pole of the facial motor nucleus. These neurons were then tested for a projection to the thoracic spinal cord by applying electrical stimulation pulses (0.5 Hz, 0.5 ms, 0.5–1.5 mA) to the spinal electrode. The antidromic nature of constant-latency spikes elicited by spinal stimulation was tested using the collision test (16). Only collision test-positive neurons were studied further. Barosensitivity of single units was tested using aortic occlusion, as described previously (37). The majority of the medullospinal neurons encountered were inhibited by arterial blood pressure elevation as expected (5). However, a number of neurons were found to be very modestly inhibited or not inhibited at all by arterial blood pressure elevation. All neurons were tested for their response to 2-DG (300 mg/kg iv), and 2-DG was administered only once to each animal. Although all unit recording experiments were performed using isoflurane anesthesia, we found that the use of this agent resulted in blood glucose concentrations that were usually 5–6 mM. On the other hand, urethane anesthesia combined with artificial respiration as described above resulted in normoglycemia (<6 mM). Therefore, the experiments in which blood glucose was measured after disinhibition of the RVLM

Fig. 1. Effect of 2-deoxyglucose (2-DG) on the discharge rate of rostroventrolateral medullary (RVLM) medullospinal neurons. A1: 2-DG activates a baroinsensitive RVLM medullospinal neuron. The discharge rate of this unit was only modestly reduced by elevation of arterial blood pressure induced by subdiaphragmatic aortic occlusion (A0c). A2: collision test for this neuron. Top trace: 3 superimposed traces in which a spontaneous spike triggers a spinal electrical stimulus that elicits a constant latency spike. Bottom trace: spinal stimulation applied within the critical interval results in collision. A2: enlargements of spontaneous (s) and antidromic (a) spikes. A3: pulse-triggered histogram of unit discharge. B1: baroinsensitive RVLM presympathetic neuron. In this example, blood pressure elevation (A0c) produced a marked decrease in discharge rate. B2: collision test for this neuron. Top trace: 3 superimposed traces in which a spontaneous spike triggers a spinal electrical stimulus that elicits a constant latency spike. Bottom trace: spinal stimulation applied within the critical interval results in collision. Note that the antidromic latency is less than that depicted for neuron in A2. B2: enlargements of spontaneous (s) and antidromic (a) spikes. C: comparison of calculated spinal axonal conduction velocities (CV) for 2-DG-responsive and 2-DG-unresponsive neurons. AP, arterial blood pressure. Data are expressed as means ± SE. *P < 0.05.
using microinjections of bicuculline were all done after cessation of isoflurane and conversion to urethane anaesthesia (1.4 g/kg iv).

*Intracerebral microinjection.* Neurons of the RVLM were activated using local microinjections of the GABA<sub>A</sub> receptor antagonist bicuculline methiodide (100 pmol/100 nl). This volume of injectate was used to ensure the spread of bicuculline throughout the RVLM. Microinjectors were made as described previously (29). To locate the RVLM accurately, we first mapped the location of the facial nucleus, using anterograde mapping as described above. Identification of a spontaneously active, barosensitive neuron whose discharge rate was markedly and reproducibly reduced by aortic cuff inflation was used to identify the stereotaxic coordinates for subsequent microinjection of bicuculline. Blood glucose was measured every 5 min in the 20 min prior to bicuculline microinjection and every 5 min thereafter for up to 1 h. Fluorescent microbeads (FluoSpheres) were incorporated into the injectate so that the injection sites in the medulla could be identified later in cryostat cut sections.

**Adrenalectomy.** Bilateral adrenalectomy or sham surgery was performed after implantation of the occlusive aortic cuff. Each gland was dissected free of surrounding tissue, and then a double ligature was placed around the nerve fibers, connective tissue, and blood vessels between the gland and the suprarenal ganglion. The adrenal glands were then removed by dividing the tissue between the ligatures. Glands were examined to verify their complete removal. Control animals received only a midline abdominal incision associated with implantation of the aortic cuff. Adrenalectomy and sham surgery were performed at least 1 h prior to microinjection of bicuculline.

**Blood glucose measurement.** Blood glucose was measured in arterial blood taken from the arterial cannula. A drop of arterial blood was placed onto the test strip of a glucometer (Optium Xceed; MediSense, Yellow Springs OH). The device is linear over the range 2.7–27.7 mM, and a Yellow Springs International Glucose Analyzer (YSI, Yellow Springs OH) was placed onto the test strip of a glucometer (Optium Xceed; MediSense, Bedford, MA). This device is calibrated against a Yellow Springs International Glucose Analyzer (YSI, Yellow Springs OH). The device is linear over the range 2.7–27.7 mM, and the interassay coefficient of variation was <5.2%.

**Materials.** 2-DG and urethane were purchased from Sigma-Aldrich (St. Louis, MO). Fluorescent microbeads (FluoSpheres) were obtained from Invitrogen Molecular Probes (Eugene, OR).

**Histological localization of injection site.** At the end of each microinjection experiment, rats were deeply anesthetized (4% isoflurane) before transcardial perfusion with phosphate-buffered saline (PBS; 0.05 M, pH 7.6) followed by 4% formaldehyde (Riedel de Haen, Seelze, Germany) in PBS. The brains were removed and stored in 4% formaldehyde-PBS until required for sectioning. Forty-micrometer-thick sections were cut at −25°C on a cryostat (Cryocut 1800; Grafe Scientific; Ringwood, Victoria, Australia) and collected onto gelatin-subbed slides and subsequently coverslipped using Vectashield mounting medium for fluorescence (Vector Laboratories, Inc, Burlingame, CA). Sections were scanned under a fluorescence microscope (Olympus BX60, Tokyo, Japan) with a wide-band green filter (bandpass 510–550 nm) for identification of the fluorescent bead injection sites. A combination of light and fluorescence microscopy provided clear anatomic delineation of brainstem areas. The sections displaying the “center” of the injection sites were photographed using an MCID imaging system (Imaging Research, ON, Canada) and a 24-bit 3-CCD color video camera (DXC-9100P; Sony, Tokyo, Japan; resolution 782 × 582 pixels). Injection sites were mapped with reference to a standard rat brain atlas (22) to verify their location within the confines of the RVLM.

**Data analysis and statistics.** Arterial blood pressure, heart rate, and extracellular action potentials were recorded using a Cambridge Electronic Design data acquisition system (CED, Cambridge, UK) and Spike2 software. Data are expressed as means ± SE. Statistical analyses were performed using a one-way ANOVA followed by a Tukey-Kramer test using GraphPad Instat v. 3.06 (GraphPad Software, San Diego, CA).

**RESULTS**

**Effect of 2-DG-induced neuroglucoprivation on RVLM medullospinal neurons.** The majority (17/23) of spontaneously active units tested for their response to neuroglucoprivation were barosensitive, spinally projecting neurons. These were either unaffected by 2-DG (8/17) or modestly inhibited (≤10%; 9/17; Fig. 1B1). These neurons had a mean conduction velocity of 2.9 ± 0.5 m/s and displayed pulse-synchronous discharge as determined by construction of arterial pulse-triggered histograms (Fig. 1B3). In contrast, we recorded a smaller group of baroinsensitive units that was prominently activated by 2-DG (Fig. 1A1). These neurons did not exhibit pulse-synchronous discharge (Fig. 1A3). The response to 2-DG appeared within ~60–90 s. These neurons had a mean spinal axonal conduction velocity of 1.1 ± 0.6 m/s (n = 6; Fig. 1C). The difference in conduction velocity noted for the two groups of neurons is apparent from the examples of the collision tests for each type of neuron depicted in Fig. 1, A2 and B2. The effects of 2-DG on arterial blood pressure were quite variable and ranged from −16 to +16 mmHg (mean = ±2 mmHg, n = 23).

**Disinhibition of RVLM neurons produces increased arterial blood pressure, tachycardia, and hyperglycemia.** To produce sustained activation of neurons in the RVLM, bicuculline methiodide (100 pmol/100 nl) was microinjected into the RVLM after electrophysiologival identification of the target area. Disinhibition of the RVLM in adrenal-intact rats produced an increase in arterial blood pressure and heart rate as expected, and this was accompanied by a marked elevation in blood glucose. In the example shown in Fig. 2A, blood glucose...
rose from 3.7 to 15.1 mM. In a group of five rats, RVLM disinhibition produced an increase in blood glucose from 4.9 ± 0.3 to 14.7 ± 0.9 mM. In contrast to the response noted in intact rats, blood glucose was unaltered in an adrenalectomized rat (Fig. 2B). In a group of adrenalectomized rats, disinhibition of the RVLM altered blood glucose from 4.2 ± 0.5 to 7.4 ± 1.6 mM (P > 0.05 vs. adrenal-intact rats, n = 5). The location of the microinjection sites is depicted in Fig. 3. All microinjection sites were found in the pressor region of the RVLM in the area within 500 μm of the caudal pole of the facial nucleus.

The group results from these experiments are shown in Fig. 4. RVLM disinhibition increased arterial blood pressure from 123 ± 6 to 178 ± 6 mmHg, whereas heart rate rose from 367 ± 20 to 417 ± 19 beats/min (P < 0.05 for both comparisons, n = 5 rats). In adrenalectomized rats, arterial blood pressure rose from 119 ± 6 to 184 ± 9 mmHg, whereas heart rate rose from 370 ± 12 to 430 ± 22 beats/min (P < 0.05 for both comparisons, n = 5 rats).

DISCUSSION

The major new finding reported in this study is the identification of a population of medullospinal neurons in the RVLM that are activated by neuroglucoprivation but are not modulated significantly by baroreceptor input. In addition, we have demonstrated that disinhibition of RVLM neurons produced by local microinjection of a GABA<sub>A</sub> receptor antagonist produced hyperglycemia along with the expected pressor and tachycardic responses. Importantly, the hyperglycemic response was abolished by adrenalectomy, but the cardiovascular responses remained unaffected. This suggests that the hyperglycemia occurs in response to epinephrine secretion. Furthermore, it suggests that the cardiovascular actions of RVLM stimulation are dependent on sympathetic vasomotor activation but not substantially on adrenomedullary catecholamine release.

Until relatively recently, the prevailing view was that RVLM neurons that controlled the sympathetic vasomotor drive had broadly similar properties to those that control adrenomedullary catecholamine secretion. In contrast, the present study suggests that, although the adrenomedullary and vasomotor premotor neurons are intermingled in the RVLM, their functional characteristics are quite different.

Morrison et al. (21) reported the existence of adrenal preganglionic neurons that had similar properties to the putative premotor neurons described in the present report and were driven by stimulation of the RVLM. These findings, along with the observations that chemical stimulation of the RVLM leads to adrenal catecholamine secretion (20), suggest that the population of neurons identified in the present study drive adrenomedullary epinephrine secretion.

RVLM barosensitive medullospinal neurons, the so-called “cardiovascular or presympathetic vasomotor neurons”, are critically involved in generation of sympathetic vasomotor outflow to the vasculature as well as in the operation of several cardiovascular reflexes, including the baroreflex, the von Bezold-Jarisch reflex, and the sympathetic chemoreflex, as well as the gastrointestinal circulatory reflex evoked by cholecystokinin (10, 11, 30, 33, 35). These neurons were not activated by 2-DG administration; indeed, some were modestly inhibited by this treatment. Therefore, it is likely that the 2-DG-responsive neurons described in the present study control the release of epinephrine, resulting in activation of liver
gycogenolysis and gluconeogenesis leading to hyperglycemia (12, 18). However, we cannot rule out a direct effect of the RVLM on liver sympathetic outflow.

While it has long been known that excitation of neurons in the RVLM activates epinephrine secretion (19), the properties of these neurons have not been described previously nor has their influence on glucose homeostasis been established previously. Indeed, it was largely assumed that the neurons responsible for sympathetic drive to the vasculature had properties that were identical to those that controlled the adrenal sympathetic outflow (23). However, this view was challenged when adrenal sympathetic preganglionic neurons that control epinephrine secretion were first identified (21). Neurons that are activated by neuroglucoprivation are clearly a minor subset of the total population of medullospinal neurons of the RVLM. These neurons were difficult to find, perhaps because they are few in number or perhaps because they discharge relatively slowly under the conditions of the experiment. These neurons may also correspond to those identified in an early study by Brown and Guyenet (4), which reported a population of sparsely projecting baroinsensitive neurons that were intermingled with the barosensitive, sparsely projecting population.

Epinephrine is an important counterregulatory hormone, particularly in individuals with type 1 and advanced type 2 diabetes (9). These patients frequently experience hypoglycemia, especially when their condition is treated aggressively (1, 8). In these circumstances, they are critically dependent on the release of epinephrine, because they cannot suppress insulin production and their ability to secrete glucagon is also impaired (7). Neuroglucoprivation induced by insulin or 2-DG is detected by glucose-sensitive neurons that are located close to the portal vein in the periphery and at several sites in the CNS (18). In the brain, glucose-sensitive neurons have been identified in several hindbrain sites as well as in the arcuate nucleus, the ventromedial hypothalamic nucleus, and the lateral hypothalamic/perifornical area (6, 28). There is a considerable body of evidence that suggests that activation of lateral hypothalamic neurons can modulate adrenal sympathetic outflow (14, 34). Thus, local neuroglucoprivation or electrical stimulation in the lateral hypothalamic area activates adrenal sympathetic outflow.

We expect that the neurons that were activated by 2-DG would also be activated by insulin-induced hypoglycemia. However, hyperinsulinemia also may activate vasomotor sympathetic outflow by activation of glutamate receptors in the RVLM (3). This suggests that its use as a tool to identify neurons that control epinephrine secretion may be limited, since some neurons may be activated in response to the ensuing neuroglucoprivation while the “cardiovascular” neurons may be activated by the hyperinsulinemia per se. We considered that testing the response of each neuron under the conditions of a hyperinsulinemic euglycemic clamp in addition to hyperinsulinemic hypoglycemia would be extremely difficult to achieve. Finally, neuroglucoprivation induced by insulin develops quite slowly, and this is a major problem when recording from single brain neurons.

At present, the phenotype of the RVLM neurons that were activated by 2-DG is unknown. Juxtacellular neuronal recording of these neurons was not attempted since 2-DG-responsive neurons were encountered infrequently. However, several observations suggest that these neurons belong to the C1 catecholamine group of the RVLM. First, destruction of these neurons using a selective catecholamine neuron neurotoxin abolishes the adrenomedullary response to neuroglucoprivation (17, 25) but not the feeding response (25). Second, neurons of the C1 subtype usually have slow-conducting spinal axons (32), and RVLM stimulation activates 2-DG-responsive adrenal sympathetic preganglionic neurons over a slow-conducting spinal pathway (21). Finally, neuroglucoprivation-induced Fos expression occurs in C1 neurons of the RVLM and in other brain locations (27). This suggests that the neurons identified in the present study may also be C1 neurons. In conclusion, the results of this study support the notion that a subpopulation of neurons in the RVLM is involved in glucose homeostasis and glucose counterregulatory responses.

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DISCLOSURES

No conflicts of interest are reported by the authors.

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