Insulin reduces excitation in gastric-related neurons of the dorsal motor nucleus of the vagus

Camille B. Blake and Bret N. Smith

Department of Physiology, University of Kentucky College of Medicine, Lexington, Kentucky

Submitted 18 June 2012; accepted in final form 21 August 2012

Blake CB, Smith BN. Insulin reduces excitation in gastric-related neurons of the dorsal motor nucleus of the vagus. Am J Physiol Regul Integr Comp Physiol 303: R807–R814, 2012. First published August 22, 2012; doi:10.1152/ajpregu.00276.2012.—The dorsal motor nucleus of the vagus (DMV) in the caudal brain stem is composed mainly of preganglionic parasympathetic neurons that control the subdiaphragmatic viscera and thus participates in energy homeostasis regulation. Metabolic pathologies, including diabetes, can disrupt vagal circuitry and lead to gastric dysfunction. Insulin receptors (IRs) are expressed in the DMV, and insulin crosses the blood-brain barrier and is transported into the brain stem. Despite growing evidence that insulin action in the brain is critical for energy homeostasis, little is known about insulin’s action in the DMV. We used whole cell patch-clamp recordings in brain stem slices to identify effects of insulin on membrane and synaptic input properties of DMV neurons, including a subset of gastric-related cells identified subsequent to injection of a retrograde label into the gastric wall. Insulin application significantly reduced the frequency of spontaneous and miniature excitatory, but not inhibitory postsynaptic currents, with no change in amplitude (P < 0.05). Insulin also directly hyperpolarized the membrane potential (~4.2 ± 1.3 mV; P < 0.05) and reduced action potential firing (P < 0.05). Insulin effects were eliminated in the presence of a ATP-dependent K⁺ (KATP) channel antagonist tolbutamide (200 μM), or the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (100 nM), suggesting that insulin inhibition of excitatory input to gastric-related DMV neurons was mediated by KATP channels and dependent on PI3K activity. Insulin regulation of synaptic input in the DMV may influence autonomic visceral regulation and thus systemic glucose metabolism.

autonomic; diabetes; glutamate; nucleus tractus solitarius; patch-clamp; vagus

INSULIN CROSSES the blood-brain-barrier and has many physiological effects centrally (5), especially on factors involved in energy homeostasis, including glucose regulation (41), food intake (6), and satiation (2). Insulin receptors and components of insulin signaling pathways are located in various brain regions involved in homeostatic regulation of metabolism, including the dorsal vagal complex (22, 23, 32, 35, 45). The dorsal vagal complex refers to a group of nuclei including the dorsal motor nucleus of the vagus (DMV), the nucleus of the tractus solitarius (NTS), the tractus solitarius, and the area postrema, located in the caudal brain stem. Neural circuits in the dorsal vagal complex integrate endocrine with peripherally and centrally arising neural signals to produce coordinated parasympathetic motor output via the vagus nerve. The DMV contains preganglionic parasympathetic motor neurons and also contains insulin receptors (23, 32, 35, 45). In particular, DMV neurons comprise the main parasympathetic preganglionic neu-
method allows for identification of preganglionic DMV motor neurons specifically related to gastric motor output. Prior recordings from similarly identified cells indicated that intrinsic cell morphology, intrinsic membrane properties, and responses to ligands binding receptor protein kinases or G protein-coupled receptors were not adversely affected by the labeling method (4, 11, 13, 19–21, 42, 46, 47).

**Brain stem slice preparation.** Brain stem slices were prepared by first anesthetizing a mouse with isoflurane, followed by decapitation. The brain was then removed and immediately immersed in ice-cold (0–4°C), oxygenated (95% O2-5% CO2) artificial cerebrospinal fluid (aCSF) containing (in mM) 124 NaCl, 3 KCl, 26 NaHCO3, 1.4 NaH2PO4, 11 mM glucose, 1.3 CaCl2, and 1.3 MgCl2, pH = 7.2–7.4, with an osmolality of 290–305 mosmol/kg H2O. The brain stem was then mounted on a metal stage, and 300 µM coronal slices were cut with a vibrotome. For consistency, slices from the caudal dorsal vagal complex near the level of the rostral area postrema (± 600 µm rostrocaudally) were used. The slices were then transferred to a holding chamber containing warmed (32–34°C) aCSF for at least 1 h. The aCSF used for recordings was identical to that used in the dissection, except when drugs were added as described.

**Patch-clamp recording.** After an equilibration period of ~1 h, whole cell voltage-clamp recordings were obtained from DMV neurons under visual guidance on an upright, fixed-stage microscope equipped with infrared illumination and differential interference contrast (IR-DIC) and epifluorescence optics (BX51WI, Olympus). Recording pipettes were pulled from borosilicate glass capillaries with 0.45 mm wall thickness (King Precision Glass, Claremont, CA). Open tip resistance was 2–5 MΩ; seal resistance was 1–5 GΩ; and series resistance was 5.6–25.5 MΩ (mean = 16.11 ± 0.68 MΩ), uncompensated. Patch pipettes were filled with (in mM) 130 KCl, 5 EGTA, 1 MgCl2, 1 CaCl2, 3 KOH, 2 ATP; pH = 7.2–7.4. Neural activity was recorded using a Multiclamp 700B patch-clamp amplifier (Molecular Devices, Axon Instruments, Sunnyvale, CA), low-pass filtered at 5 kHz, acquired using a Digidata 1440A digitizer and pClamp 10.3 software. Synaptic currents were analyzed off-line on a PC-style computer with pCLAMP programs (Axon Instruments) or MiniAnalysis 6.0.3 (Synaptosoft, Decatur, GA). Synaptic currents had a fast (<1 ms) rise time and exponential decay. A value of twice the root mean squared noise level for a given recording was used as the detection level for synaptic current amplitude. Inhibitory and excitatory postsynaptic currents (IPSCs and EPSCs, respectively) were recorded in voltage-clamp mode, while action potentials (APs) and input resistance were recorded at rest in current-clamp mode.

**Drug application.** Added to the aCSF for specific experiments were the following: insulin (0.1, 0.3, 0.5, 1.0, or 3.0 µM, Sigma-Aldrich, St. Louis, MO), the PI3K inhibitor wortmannin (100 nM, Sigma; 47), the Na+ channel blocker tetrodotoxin (TTX; 2 µM, Tocris Bioscience, Minneapolis, MN), the KATP channel blocker tolbutamide (200 µM; Sigma, 47), the competitive AMPA/kainate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 µM, Sigma), the selective NMDA receptor antagonist dl-2-amino-5-phosphono-pentanoic acid (AP-5; 100 µM; Sigma), and/or the noncompetitive GABA_A receptor antagonist picROTOX (100 µM; Sigma). Incubation times for agonists (i.e., insulin) was 2–10 min; antagonists and channel blockers were preapplied for at least 15 min before and during agonist application.

**Analysis.** Once in the whole cell configuration, cells were voltage clamped initially near the resting membrane potential (determined by measuring the voltage at which I = 0) for 10 min to allow equalization of the intracellular milieu and recording electrode solutions. Resting membrane potentials were corrected post-hoc using a Junction Potential Calculator (pClamp 10.3 software; Axon Instruments). EPSCs were examined at a holding potential of ~70 mV, while IPSCs were examined at 0 mV. For spontaneous and miniature synaptic currents, at least 2 min of continuous activity (typically 500–2,500 events) were examined to identify insulin effects on amplitude and frequency distributions. The in-situ Kolmogrov-Smirnov test was

![Image](https://placehold.it/150x150)
used to determine drug effects on PSC frequency and amplitude within a recording. Effects of insulin on mean input resistance, resting membrane potential, PSC frequency, amplitude, rise, and decay time constants were determined using a two-tailed, paired Student’s t-test.

**RESULTS**

**Insulin effects on spontaneous excitatory synaptic transmission.** To assess insulin effects on excitatory synaptic transmission, DMV neurons were voltage-clamped at −70 mV. Insulin significantly reduced the frequency of spontaneous EPSCs (sEPSCs) in 10 of 12 neurons (from 23.8 ± 5.7 Hz in control aCSF to 13.8 ± 2.8 Hz in insulin; 38% mean decrease; n = 12; P < 0.05; Fig. 1). There was no significant change in sEPSC amplitude (35.0 ± 3.4 pA in control aCSF to 34.3 ± 4.3 pA in insulin; P = 0.91; Fig. 1). Application of different concentrations of insulin (100 nM-3 μM) resulted in a concentration-dependent decrease in sEPSC frequency (Fig. 1D). The maximum response was observed with 1 μM insulin, so this concentration was used for analyses of further effects of insulin.

To determine whether the effect of insulin was due to activation of the K<sub>ATP</sub> channel, insulin was applied in the presence of tolbutamide. In the presence of tolbutamide, application of insulin failed to produce a significant change in the frequency of sEPSCs (from 6.0 ± 1.3 Hz in tolbutamide to 5.2 ± 1.0 Hz in tolbutamide + insulin; n = 6; P > 0.05; Fig. 1D). The frequency of sEPSCs in cells treated with tolbutamide (6.0 ± 1.3 Hz) was not significantly different from the sEPSC frequency in control aCSF (23.8 ± 5.7 Hz; between cells; P = 0.07). Thus the insulin-induced decrease in sEPSC frequency was eliminated when cells were preincubated in tolbutamide.

To determine whether the effect of insulin on sEPSC frequency was dependent on PI3K activity, insulin was applied in the presence of wortmannin. When insulin was applied in the presence of wortmannin, there was no significant change in sEPSC frequency (from 6.3 ± 2.1 Hz in wortmannin to 5.7 ± 1.9 Hz in wortmannin + insulin; n = 8; P > 0.05; Fig. 1E). The frequency of sEPSCs in cells treated with wortmannin (6.3 ± 2.1 Hz) was not significantly different from the sEPSC frequency in control aCSF (23.8 ± 5.7 Hz; between cells; P = 0.07). Thus the insulin-induced decrease in sEPSC frequency was eliminated when cells were preincubated in wortmannin.

To determine whether insulin effects occurred on neurons that regulate the gastric musculature, the effect of insulin was determined in recordings from neurons that expressed mRFP1 after PRV-614 inoculation of the stomach (Fig. 1, inset) (20, 46). Insulin (1 μM) decreased sEPSCs of 6 of 8 neurons expressing mRFP1 (from 10.2 ± 2.4 in control to 6.6 ± 1.9 in insulin; 36% mean decrease; n = 8; P < 0.05; Fig. 1D), with no effect on amplitude (25.0 ± 1.9 in control to 22.7 ± 1.6 in insulin; n = 8; P = 0.16; Fig. 1E). The insulin-induced decrease in sEPSC frequency in this subset of gastric-related DMV neurons was similar to that for unidentified DMV neurons.

**Insulin effects on miniature excitatory synaptic transmission.** Frequency, but not amplitude, of sEPSCs was altered by insulin, suggesting effects on synaptic release and/or afferent activity. To determine whether the effects of insulin were mediated by
receptors on presynaptic terminals, DMV neurons were voltage-clamped at a holding potential of −70 mV and miniature EPSCs (mEPSCs) were recorded in the presence of TTX (2 μM), which blocks AP-dependent synaptic activity. Insulin significantly reduced the frequency of mEPSCs in 7 of 10 neurons (from 4.40 ± 1.02 Hz in TTX to 1.6 ± 0.8 Hz in TTX + insulin; 64% overall decrease; n = 10; P < 0.05; Fig. 2), with no change in amplitude (P = 0.69; n = 10). These data suggested that insulin acts on insulin receptors located on glutamatergic afferent terminals synapsing on DMV neurons.

**Effects of insulin on spontaneous inhibitory synaptic transmission.** To examine the effects of insulin on inhibitory synaptic transmission in the DMV, spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded while voltage-clamping DMV neurons at 0 mV. Insulin (1 μM) did not produce a change in the frequency of sIPSCs in any neuron. Mean sIPSC frequency was 2.3 ± 0.4 Hz in control aCSF and 2.3 ± 0.8 Hz in the presence of insulin (n = 6; P > 0.05; Fig. 3). Unlike excitatory synaptic activity, insulin did not affect inhibitory transmission in DMV neurons.

**Effects of insulin on membrane properties.** To analyze insulin effects on membrane potential, input resistance, and AP firing, DMV neurons were recorded in current-clamp mode. Neurons recorded in voltage clamp were also periodically monitored for changes in membrane potential by periodically removing the voltage clamp (i.e., switch to I = 0). Bath application of insulin (1 μM) resulted in a membrane hyperpolarization of 4.2 ± 1.3 mV from the resting membrane potential (−54.3 ± 1.4 mV) in 15 of 18 unidentified DMV neurons (P = 0.001, n = 18; Fig. 4). In the remaining three neurons, the membrane potential was either depolarized slightly (from −53.5 ± 2.5 mV to −49.5 ± 1.5 mV; n = 2) or unchanged (−57.5 to −58.0; n = 1). Input resistance was measured in six of the cells that were hyperpolarized. Input resistance was significantly decreased by insulin in all six of these neurons (749.8 ± 48.6 MΩ in control to 655.3 ± 56.3 MΩ in the presence of insulin; n = 6; P < 0.05).

In the presence of TTX, a hyperpolarization (−4.7 ± 1.9 mV) was induced by insulin in 7 of 10 neurons (from −54.1 ± 2.6 mV in TTX to −59.6 ± 2.5 mV in TTX + insulin; n = 10), indicative of a direct membrane hyperpolarization, independent of AP-mediated synaptic transmission. Input resistance was decreased in 6 of these 7 neurons. Mean input resistance in the cells that displayed a hyperpolarization in the presence of TTX was 766.1 ± 60.8 MΩ in control and 636.6 ± 64.8 MΩ in insulin (n = 7; P < 0.01; Fig. 4). Thus insulin significantly affected resting membrane potential and whole cell input resistance.

To investigate insulin effects on spontaneous AP firing of DMV neurons, cells that fired spontaneous APs were examined at the resting membrane potential (−54.3 ± 1.4 mV; n = 10). Mean AP frequency was 2.2 ± 0.3 Hz. Insulin significantly reduced the frequency spontaneous AP firing in 9 of 10 DMV neurons (from 2.2 ± 0.3 Hz in aCSF to 1.5 ± 0.2 Hz in insulin; 27% mean decrease; P < 0.05; n = 10; Fig. 5). The effect of insulin was still present after 15 min of washout in normal aCSF (Fig. 5D). Insulin (1 μM) also significantly decreased spontaneous AP firing in 4 of 5 neurons expressing mRFP1 after PRV-614 inoculation of the stomach (from 1.27 ± 0.17 Hz in control to 0.77 ± 0.10 Hz in insulin; 39% decrease; n = 5; P < 0.05; Fig. 5D).

To block synaptic currents, neurons were incubated in a solution containing CNQX (10 μM), AP-5 (100 μM), and picrotoxin (100 μM). In the presence of these synaptic blockers, insulin application produced a decrease in AP frequency of similar magnitude to that seen in control aCSF (2.8 ± 0.7 Hz in synaptic blockers to 2.2 ± 0.6 Hz in synaptic blockers + insulin; n = 7; 28% mean decrease; P < 0.01; Fig. 5D). These results suggested direct effects of insulin on AP frequency in DMV neurons, independent of effects on fast synaptic transmission.

The decrease in AP frequency was consistent with a PI3K-dependent activation of the KATP channel, similar to previous reports on the effects of insulin in the brain (14, 15, 36, 37, 43). When insulin (1 μM) was applied in the presence of the KATP channel antagonist tolbutamide, there was no significant change in AP frequency (from 2.2 ± 0.9 Hz in tolbutamide to 3.0 ± 1.0 Hz in tolbutamide + insulin; n = 6; P = 0.29; Fig. 5D) or membrane potential (from −49.6 ± 3.0 mV in tolbutamide to −51.3 ± 2.9 mV; n = 6; P = 0.70). Likewise, there was no significant change in AP frequency when insulin was applied in the presence of the PI3K inhibitor wortmannin (from 0.7 ± 0.1 Hz to 0.6 ± 0.1 Hz; n = 6; P = 0.41).

---

Fig. 3. Insulin did not affect spontaneous inhibitory postsynaptic currents (IPSCs). Sample traces of spontaneous IPSCs observed at a holding potential of 0 mV in a DMV neuron in control aCSF (A) and insulin (1 μM) (B). Bottom traces are expanded portions of outlined areas (500 ms). C: cumulative probability plot for the displayed recordings. D: insulin did not produce a significant change in the frequency of sIPSCs in DMV neurons (P = 0.97; n = 5).
Hz in wortmannin to 0.9 ± 0.3 Hz in wortmannin + insulin; n = 7; P = 0.20; Fig. 5D), or in membrane potential (from −55.1 ± 1.1 mV in wortmannin to −55.4 ± 1.2 mV in wortmannin + insulin; n = 7; P = 0.65). Thus the insulin-induced decrease in AP frequency and membrane hyperpolarization were eliminated when K\textsubscript{ATP} channels or PI3K activity were blocked.

**DISCUSSION**

This study showed that insulin reduced excitatory activity of DMV neurons, including in a subset of gastric-projecting neurons, likely acting at sites both pre- and postsynaptically. Insulin inhibited glutamatergic excitatory synaptic input and also caused a membrane hyperpolarization and decrease in AP frequency. The effects of insulin were eliminated by the application of the PI3K inhibitor wortmannin or the K\textsubscript{ATP} channel antagonist tolbutamide, suggesting that insulin acts on the insulin receptor pathway seen in inhibitor wortmannin or the K\textsubscript{ATP} channel antagonist tolbutamide, suggesting that insulin is at receptors located on glutamatergic synaptic terminals within the slice.

The location of neurons supplying input that was affected by insulin is not certain, but glutamatergic NTS neurons that project to the DMV have been identified (10), and direct primary afferent input to DMV neurons has also been proposed (39). Effects of insulin on somato-dendritic regions of afferent neurons (e.g., in the NTS) or on primary viscerosensory afferent terminals remain to be determined. The most prominent input to the DMV arising from the NTS is likely to be GABAergic, at least in rats (10, 44). Recordings herein were made from murine cells, and glutamatergic EPSCs are typically more frequent in mice than in rats, suggesting a possible species-dependent increased influence of these inputs. Several substances can modulate GABAergic input to the DMV under basal conditions (11–13). However, a number of molecules are reported to selectively modulate glutamate, but not GABA, release in the DMV under basal conditions. Many of these have been found to also alter GABA release, but only after additional “priming” of the system induced by variations in second messengers like cAMP (7). Thus modulation of GABAergic input from the NTS may in some cases exist “on demand,” being determined by the activity of other molecules. Like insulin, leptin also selectively inhibited glutamate release in the DMV, with little effect on GABAergic activity (47), suggesting functional overlap of responses to these energy-sensing molecules. The hypothesis that insulin might also modulate GABA release after second messenger “priming” represents a potentially interesting avenue of study to determine whether responses to insulin are energy state dependent.
Insulin also induced a membrane hyperpolarization accompanied by a decrease in whole cell input resistance in the majority of DMV cells tested and decreased the frequency of spontaneous action potential firing. The effects on AP frequency persisted when synaptic input was blocked pharmacologically, suggesting direct effects on DMV neurons, independent of effects on synaptic input. Notably, all of the observed insulin effects were blocked by tolbutamide and wortmannin, consistent with effects mediated by PI3K-dependent KATP channels, regardless of whether presynaptic or postsynaptic elements are involved.

Previous studies have shown that reducing or increasing glucose levels on brain stem slices results in alterations of the K<sub>ATP</sub> current (3, 16). Hyperglycemia associated with diabetes also causes severe alterations in sulfonylurea receptor binding sites in the brain (30). The sulfonylurea receptor is an integral part of the K<sub>ATP</sub> channel that is modulated by glucose, leptin, and insulin. Similar to the effects of leptin (46) and glucose (18, 26), the effects of insulin observed here were sensitive to K<sub>ATP</sub>-channel blockade and PI3K inhibition, suggesting overlap in their effects and mechanisms. Thus hyperglycemia results in altered cellular signaling in the brain stem. Understanding potential interactions between insulin and the effects of glucose or leptin in this system may provide further insight into the regulation of parasympathetic function in metabolic diseases.

Brief glucose elevation in the dorsal vagal complex in the absence of insulin (16) or longer-term hyperglycemia when insulin is reduced (48) leads to downstream effects on gastric motility and tone and persistent effects on vagal motor neuron function. Additionally, gastrointestinal dysfunction due to vagal dysregulation has been reported in diabetic patients (40). In a model of Type-1 diabetes, mice treated with streptozotocin (STZ) to destroy insulin-producing β cells in the pancreas, the frequency of mEPSCs in the DMV was significantly increased over that of control mice. Furthermore, TRPV1 receptors on glutamate terminals were internalized in this model, a form of plasticity that was reversed by insulin application (48), suggesting long-term effects of removing endogenous insulin. Additionally, Gelling et al. (17) demonstrated that insulin receptor substrate (IRS)-PI3K signaling in the hypothalamus is disrupted in STZ-treated animals with chronic hyperglycemia, which is also reversed by insulin treatment (17). Insulin applied in the hypothalamus or third ventricle can improve hepatic neoglucogenesis in models of diabetes, and this effect is vagally mediated (33, 34, 38). Insulin has previously been found to stimulate glucose uptake in the dorsal vagal complex (3). Systemic effects of insulin in the dorsal vagal complex could thus also include indirect actions on glucose utilization, in addition to the direct effects observed here. Insulin action within the brain helps regulate glucose levels in the periphery, and, based on the present findings and previous reports, the dorsal vagal complex likely participates in this circuit.

Previous studies have shown vagally mediated visceral effects due to insulin action in the dorsal vagal complex. For instance, insulin microinjected into the dorsal vagal complex produces increases in intragastric pressure, pyloric and curvature contractile...
activity, and blood pressure (28). Additionally, insulin injected directly into the NTS of anesthetized rats produces depressor and bradycardic effects, which are dependent on PI3K (25). While insulin-induced hypoglycemia enhances vagal activity (24), hyperglycemia depresses vagal tone (31). Therefore, insulin appears to act in the dorsal vagal complex to potentially decrease gastric tone. The action of insulin observed here is consistent with these effects because it inhibits excitatory activity of DMV motor neurons that contribute to vagally mediated output. Diminishing excitation by insulin might be expected to have effects qualitatively similar to increasing inhibition in the dorsal vagal complex (e.g., by glucose; 16) with regard to decreasing gastric tone. Indeed, insulin may have additional effects on glucose utilization in the dorsal vagal complex or on glucose sensitivity, though this hypothesis requires further testing.

Perspectives and Significance

In the current study, we found that insulin decreases excitatory activity in the DMV in two ways. In addition to effects at receptors located on the postsynaptic membrane that lead to changes in membrane potential, the effect on mEPSCs suggests that insulin also acts at receptors on presynaptic glutamatergic terminals contacting DMV neurons. This suggests multiple means for insulin to affect parasympathetic motor function, including both direct effects on the DMV cells and by modulation of conditioning synaptic input. We believe that insulin may inhibit gastric tone by decreasing excitatory activity of DMV neurons. Identifying insulin effects in the context of metabolic dysfunction or nutrient alterations that could affect synaptic relationships in the dorsal vagal complex may provide insight into central mechanisms contributing to diabetes, obesity, and energy homeostasis.

GRANTS

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants R01 DK-056132 and F32 DK-089717.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.B.B. and B.N.S. conception and design of research; C.B.B. and B.N.S. performed experiments; C.B.B. and B.N.S. analyzed data; C.B.B. and B.N.S. interpreted results of experiments; C.B.B. and B.N.S. prepared figures; C.B.B. drafted manuscript; C.B.B. and B.N.S. edited and revised manuscript; C.B.B. and B.N.S. approved final version of manuscript.

REFERENCES