cAMP-dependent insulin modulation of synaptic inhibition in neurons of the dorsal motor nucleus of the vagus is altered in diabetic mice

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Blake CB, Smith BN. cAMP-dependent insulin modulation of synaptic inhibition in neurons of the dorsal motor nucleus of the vagus is altered in diabetic mice. Am J Physiol Regul Integr Comp Physiol 307: R711–R720, 2014. First published July 2, 2014; doi:10.1152/ajpregu.00138.2014.—Pathologies in which insulin is dysregulated, including diabetes, can disrupt central vagal circuitry, leading to gastrointestinal and other autonomic dysfunction. Insulin affects whole body metabolism through central mechanisms and is transported into the brain stem dorsal motor nucleus of the vagus (DMV) and nucleus tractus solitarius (NTS), which mediate parasympathetic vagal motor regulation. The NTS receives viscerosensory vagal input and projects heavily to the DMV, which supplies parasympathetic vagal motor output. Normally, insulin inhibits synaptic excitation of DMV neurons, with no effect on synaptic inhibition. Modulation of synaptic inhibition in DMV, however, is often sensitive to cAMP-dependent mechanisms. We hypothesized that an effect of insulin on GABAergic synaptic transmission may be uncovered by elevating cAMP levels in GABAergic terminals. We used whole cell patch-clamp recordings in brain stem slices from control diabetic mice to identify insulin effects on inhibitory neurotransmission in the DMV in the presence of forskolin to elevate cAMP levels. In the presence of forskolin, insulin decreased the frequency of inhibitory post synaptic currents (IPSCs) and the paired-pulse ratio of evoked IPSCs in DMV neurons from control mice. This effect was blocked by brefeldin-A, a Golgi-disrupting agent, or indinavir, a GLUT4 blocker, indicating that protein trafficking and glucose transport were involved. In streptozotocin-treated, diabetic mice, insulin did not affect IPSCs in DMV neurons in the presence of forskolin. Results suggest an impairment of cAMP-induced insulin effects on GABA release in the DMV, which likely involves disrupted protein trafficking in diabetic mice. These findings provide insight into mechanisms underlying vagal dysregulation associated with diabetes.

diabetes; GABA; nucleus tractus solitarius; patch-clamp; vagus

GLUCOSE HOMEOSTASIS IS REGULATED by the brain in conjunction with the autonomic and neuroendocrine systems. Metabolic (e.g., glucose, insulin) and viscerosensory signals are integrated in the brain stem dorsal vagal complex (DVC), which then provides for parasympathetic motor control of visceral organs, including the pancreas, liver, intestines, and stomach. The DVC also has reciprocal connections with hypothalamic nuclei that are critically involved in energy homeostasis. Alteration of neural function in the DVC has long been implicated in the regulation of whole body glucose regulation (47, 48).

Diabetes mellitus is a metabolic disorder that results in dysfunctional glucose regulation via insulin deficiency (Type 1 diabetes), or resistance (Type 2 diabetes). Diabetic patients experience vagally mediated gastric dysfunction, which is dependent on brain stem activity (27, 28, 31, 32, 49). Insulin action in the brain influences many factors involved in energy homeostasis, including hepatic glucose production (22, 42, 46, 50), food intake (10), and satiety (5). At least some of these actions occur in the DVC, where insulin directly modulates vagal motor neuron activity (8). The brain, in particular, the DVC, is both affected by and contributes to blood glucose dysregulation associated with diabetes.

The DVC comprises the dorsal motor nucleus of the vagus (DMV), the nucleus of the solitary tract (NTS), and the area postrema. Within the DVC, the synaptic connections between the NTS and DMV are critical to coordinating appropriate vagal outputs. Two primary types of connections between these nuclei include the major inhibitory (GABA) and excitatory (glutamate) neurotransmitters in the brain (17, 52). Synaptic input to DMV neurons has profound effects on vagal output (53). In particular, GABAergic input exerts potent effects on DMV neuron activity and, consequently, vagal motor output (53). Because cAMP levels in GABAergic terminals are purported to be relatively low in the DMV, peptide modulation of GABA release onto DMV neurons is often without effect unless cAMP levels in GABAergic terminals are elevated (11–13, 15). Elevation of cAMP that enables neuro-modulation by these substances can be accomplished by a variety of neuroactive substances (14) or by selectively cutting primary viscerosensory vagal afferents (15). cAMP levels in GABAergic terminals, therefore, importantly regulate peptidergic neuromodulatory effects on GABA release, and thus also affect vagal activity.

We previously found that insulin hyperpolarizes vagal motor neurons and decreases synaptic excitation, but not inhibition, in the DMV of control mice (8). On the basis of evidence that other peptides can alter inhibition in the DVC in the presence of elevated cAMP (11–13), we tested the effects of insulin application on synaptic inhibition in the presence of elevated cAMP levels using whole cell patch-clamp recordings from DMV neurons in brain stem slices. Similar studies were performed in a mouse model of Type 1 diabetes to test the hypothesis that chronic hyperglycemia/hyperinsulinemia alters cAMP-dependent synaptic plasticity in the DMV.

MATERIALS AND METHODS

Animals. Whole cell patch-clamp recordings were performed using coronal brain stem slices containing the DVC from young (3–6 wk) male and female mice (CD-1, Harlan, Indianapolis, IN; or FVB, Jackson Laboratories, Bar Harbor, ME). Mice were housed in a vivarium under a normal 14-h light/10-h dark cycle with food and water available ad libitum. All animal procedures were approved by The University of Kentucky Animal Care and Use Committee.

Streptozotocin treatment. A cohort of mice (male and female) was injected with streptozotocin (STZ; 200 mg/kg; Alexis Biochemicals, San Diego, CA) to kill pancreatic β-cells and induce chronic hyper-
glycemia (>300 mg/dl blood glucose). Mice were injected intraperitoneally with STZ dissolved in sterile saline. Body weight and blood glucose levels (One Touch Ultra) were monitored prior to injection and then daily afterward. Readings maximize at 600 mg/dl, so any reading above this level was assigned a value of 600 mg/dl. Only mice with blood glucose levels >300 mg/dl (16.6 mM) for at least 3 consecutive days were considered hyperglycemic and, thus, were included in the experiments.

**Brain stem slice preparation.** Brain stem slices were prepared by first anesthetizing a mouse with isoflurane by inhalation to effect, followed by rapid decapitation. The brain was then rapidly 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. For some recordings, 2.5 mM glucose was used in the ACSF, with 8.5 mM sucrose added to maintain balanced osmolality. The brain stem was mounted on a metal stage, and 30-μM coronal (i.e., transverse) slices were cut with a vibrotome. For consistency, slices from the caudal DVC 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.

**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, Center Valley, PA). 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 4–25 MΩ (mean = 10.7 ± 0.6 MΩ), uncompensated. Pipettes were filled with (in mM): 140 Cs⁺-glucionate, 1 NaCl, 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, Sunnyvale, CA), low-pass filtered at 5 kHz, and acquired using a Digidata 1440A digitizer and pClamp 10.3 software (Molecular Devices). Synaptic currents were analyzed off-line on a PC-style computer with pCLAMP programs (Molecular Devices) 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 mean root mean square level for a given recording was used as the detection limit for synaptic current amplitude. Inhibitory postsynaptic currents (IPSCs) were recorded in voltage-clamp mode.

**Electrical stimulation.** Electrical stimulation was performed using a platinum-iridium concentric bipolar electrode (125-μm diameter, FHC, Bowdoinham, ME) placed in the medial NTS. A minimum of 15 paired current pulses (20–200 μA; 300 μs) with an interpulse interval of 100 ms and a cycle rate of 0.1 Hz were administered to the NTS. Responses in DMV neurons voltage-clamped at 0 mV were recorded and analyzed for current amplitude before, during, and after the application of forskolin and insulin.

**Drug application.** Added to the ACSF for specific experiments were kynurenic acid (1 mM; 5–60 min; Sigma Aldrich, St. Louis, MO), CNQX (10 μM; 15–45 min; Sigma Aldrich), AP-5 (100 μM; 15–45 min; Sigma Aldrich), tetrodotoxin (2 μM; 10–60 min; Alomone Laboratories, Jerusalem, Israel), brefeldin-A (5 μM; 10–20 min; Sigma Aldrich), indinavir (50 μM; 5–15 min; Sigma Aldrich) forskolin (10 μM, 5–10 min; Tocris Bioscience, Minneapolis, MN), 1,9-dideoxyforskolin (10 μM, 5–10 min; Sigma Aldrich), and/or insulin (1 μM; 5–7 min; Sigma Aldrich; cat. no. I9278).

**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 pA) for 10 min to allow equilibration of the intracellular milieu and recording electrode solutions. Membrane potential was voltage-clamped at 0 mV to examine IPSCs. For spontaneous and miniature IPSCs (i.e., sIPSCs and mIPSCs), at least 2 min of continuous activity (typically 500–2,000 events) were examined to identify insulin effects on amplitude and frequency distribution. The intra-assay, nonparametric Kolmogrov-Smirnov test was used to determine drug effects on IPSC frequency and amplitude within a recording. Effects of insulin on mean IPSC frequency, amplitude, rise time, and decay time constants were determined using a two-tailed, paired Student’s t-test or ANOVA. The paired-pulse ratio (PPr) was calculated as the ratio of the amplitude of the second evoked IPSC (eIPSC) to that of the first eIPSC. The square of the coefficient of variation (CV²) was calculated as the variance of the mean peak eIPSC amplitude divided by the square of the mean eIPSC amplitude (20).

**RESULTS**

We recently reported that insulin inhibited the membrane potential directly and suppressed glutamatergic excitatory neurotransmission in DMV neurons in a concentration-related fashion but had no effect on GABA release in the DMV under normal conditions (8). Here, all recordings were made with Cs-based internal recording solutions to block K⁺ conductances and, therefore, prevent direct effects of insulin on the neuronal membrane of the recorded cell, which were previously shown to be K⁺-dependent. In addition, either kynurenic acid (1 mM) or a combination of CNQX (10 μM) and AP-5 (100 μM) were included in the ACSF to block ionotropic glutamate receptors. Recorded neurons were voltage-clamped at 0 mV to isolate insulin effects on IPSCs. Insulin was applied at 1 μM, the concentration previously shown to have maximal effects on glutamate release in the DMV and on membrane potential of DMV neurons in brain stem slices (8). This concentration is slightly higher than maximal concentrations used previously in similar studies of insulin effects in hypothalamic slices (35, 51, 56), but differences in factors contributing to drug penetration in slices (e.g., slice thickness, cell density), drug supplier, receptor concentration, and other factors could contribute to variance in drug concentration responses. Because elevating cAMP levels with forskolin was previously shown to uncover modulatory effects of peptides and other neuromodulators on GABA release in the DMV (11–13), the effects of insulin on sIPSC frequency and amplitude in DMV neurons were assessed in the presence of the adenylate cyclase agonist, forskolin (10 μM). There were no differences between male and female mice in any of the parameters measured (data not shown). Therefore, results from both male and female mice were pooled for the analysis detailed below.

**Effects of forskolin and insulin on spontaneous inhibitory synaptic transmission.** As reported previously, insulin did not change sIPSC frequency in DMV neurons under standard recording conditions (8). The addition of forskolin (10 μM) for 5–10 min significantly increased the frequency of sIPSCs in 11 of 13 cells (3.9 ± 0.9 Hz, control ACSF: to 7.5 ± 1.6 Hz, forskolin: 92% mean increase; n = 13; P < 0.02; Fig. 1). Application of an inactive forskolin analog, 1,9-dideoxyforskolin (10 mM), was without effect on sIPSC frequency or amplitude (1.9 ± 0.9 Hz, control, 1.4 ± 0.5 Hz, 1,9-dideoxyforskolin; n = 7; P > 0.05; Fig. 1). The effect of forskolin was not blocked by pretreatment for 20 min with the Golgi-disrupting compound, brefeldin-A (5 μM; 2.7 ± 1.0 Hz,
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brefeldin-A; 4.8 ± 1 Hz, brefeldin-A + forskolin; n = 6; P < 0.003). Likewise, the addition of indinavir (50 μM), an antagonist to the insulin-induced glucose transporter 4 (GLUT4), was also ineffective in preventing the effect of forskolin on sIPSC frequency (2.3 ± 1 Hz, indinavir; 4.0 ± 1 Hz, indinavir + forskolin; n = 6; P < 0.01; Fig. 1). There was no significant change in sIPSC amplitude with forskolin alone (40.8 ± 1.9 pA, control ACSF; 42.7 ± 2.4 pA, forskolin; n = 13; P = 0.34), in the presence of brefeldin-A (37.7 ± 5.0 pA, brefeldin-A; 32.1 ± 3.1 pA brefeldin-A + forskolin; n = 6; P = 0.07) or the addition of indinavir (37.2 ± 6.1 pA, indinavir; 33.2 ± 4.2 pA indinavir + forskolin). Thus, forskolin significantly increased the frequency of sIPSCs, and this effect was not blocked by preventing protein trafficking with brefeldin-A or inhibiting GLUT4 activity with indinavir.

To determine insulin effects on sIPSCs in the presence of elevated cAMP levels, slices were treated with forskolin (10 μM) for 5 min prior to the addition of insulin (1 μM) in the presence of forskolin. Cells that did not initially respond to forskolin were not included in the analysis. Insulin significantly reduced the frequency of sIPSCs in 12 of 16 neurons in the presence of forskolin (6.8 ± 1.2 Hz, forskolin; 4.9 ± 0.9 Hz, forskolin + insulin; 28% mean decrease; n = 16; P < 0.02; Fig. 2). Application of insulin in the presence of an inactive forskolin analog, 1,9-dideoxyforskolin (10 mM), was without effect on sIPSC frequency or amplitude (1.4 ± 0.5 Hz, 1,9-dideoxyforskolin; 1.2 ± 0.2 Hz, 1,9-dideoxyforskolin + insulin; n = 7; P > 0.05; Fig. 2). The decrease in sIPSC frequency was prevented when slices were pretreated with brefeldin-A for 10–20 min (4.8 ± 1.1 Hz, forskolin + brefeldin-A; 4.7 ± 0.8 Hz with insulin added; n = 6; P = 0.85; Fig. 2). Likewise, pretreatment with indinavir also prevented the insulin-induced suppression of sIPSC frequency in the presence of forskolin (4.0 ± 1.0 Hz, forskolin + indinavir; 4.6 ± 0.9 Hz, with insulin; n = 6; P = 0.06; Fig. 2). There was no significant change in sIPSC amplitude with insulin application (42.2 ± 1.7 pA, forskolin; 39.6 ± 2.3 pA, forskolin + insulin; n = 18; P = 0.20), when pretreated with brefeldin-A (32.1 ± 3.1 pA in brefeldin-A + forskolin; 30.9 ± 3.3 pA, with insulin added; n = 6; P = 0.46) or when pretreated with indinavir (33.2 ± 4.2 pA, indinavir + forskolin; 30.9 ± 3.8 pA with insulin added).

Insulin-dependent glucose sensitivity could contribute to an effect on GABA release (21). To determine whether insulin’s effect could be modified by reducing glucose concentration, the effects of forskolin and insulin were also tested in ACSF containing 2.5 mM glucose, which yielded results similar to those observed in 11 mM glucose. In the presence of 2.5 mM glucose, forskolin signifi- cantly increased sIPSC frequency in 9 of 11 cells (3.2 ± 0.6 Hz, control ACSF; 5.4 ± 1.2 Hz, forskolin; 72% mean increase; n = 11; P < 0.003; Fig. 1). In the presence of forskolin, insulin significantly decreased the frequency of sIPSCs in 7 of 9 cells (6.2 ± 1.3 Hz, forskolin; 4.7 ± 1.1 Hz, forskolin + insulin; 24% mean decrease; n = 9; P < 0.009; Fig. 2) with no change in amplitude (39.1 ± 2.6 pA, forskolin; 38.9 ± 2.2 pA, forskolin + insulin; n = 9; P = 0.94). Similar to the effects in 11 mM glucose, insulin decreased sIPSC frequency in the presence of forskolin in ACSF containing 2.5 mM glucose.

Because forskolin induced an increase in sIPSC frequency, we determined whether the effect of insulin in forskolin was dependent on background sIPSC frequency. Regression analysis revealed that there was no correlation between the sIPSC frequency in forskolin and the degree to which insulin decreased frequency (R² = 0.19; n = 18). Thus, insulin significantly decreased the frequency of sIPSCs in the presence of forskolin to a similar degree in both 11 mM and 2.5 mM glucose-containing ACSF. This effect was not correlated with background sIPSC frequency and was prevented when receptor trafficking was blocked by brefeldin-A or in the presence of indinavir to block GLUT4 activity.

Effects of forskolin and insulin on action potential-independent GABA release. To assess forskolin effects on miniature inhibitory synaptic transmission, DMV neurons were voltage-clamped at 0 mV in the presence of glutamate receptor antagonists and tetrodotoxin (TTX; 2 μM) to block action potentials. Forskolin application significantly increased the frequency of mIPSCs in 8 of 10 cells (2.9 ± 0.7 Hz, control ACSF; 4.3 ±
Forskolin

Forskolin + Insulin

C

D

E

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Fig. 2. Insulin significantly decreased sIPSC frequency in the presence of forskolin in DMV neurons from normoglycemic mice. Representative traces from a DMV neuron in forskolin (10 μM) (A) and forskolin + insulin (1 μM) (B). C: cumulative probability plot showing the effect of insulin on sIPSC interevent interval in the presence of forskolin in recordings from the neuron shown in A and B. In the presence of forskolin, insulin significantly decreased sIPSC frequency in this neuron (P < 0.05). D: insulin significantly decreased sIPSC frequency in ACSF containing normal (11 mM) glucose and forskolin (P < 0.02), and in low (2.5 mM) glucose (P < 0.009), but not when pretreated with either the G oligo-disrupting agent, breflidil-A (P > 0.05), or with the GLUT4 antagonist indinavir (n = 6; P > 0.05). When insulin was applied in the presence of 1–9-dideoxyforskolin (10 μM), there was no effect on IPSC frequency (n = 7; P > 0.05). *Significant difference from relative control ACSF; †Significant difference from forskolin.

Effects of forskolin and insulin on GABA release in diabetic mice. A subset of mice was treated with streptozotocin to induce hyperglycemia and hypoinsulinaemia for at least three consecutive days (4–10 days; mean 6.8 ± 0.5 days; n = 13). Mean blood glucose concentration rose from 175.2 ± 3.9 mg/dl (range 155–204 mg/dl) prior to STZ injection to 557.4 ± 12.9 mg/dl (range 486–600+ mg/dl) on the day of electrophysiological experiments (P < 0.05). Mean body weight change was +5.5% (18.7 ± 0.9 g on injection day to 19.7 ± 0.8 g on experiment day). To assess the effects of insulin on inhibitory neurotransmission in diabetic animals, DMV neurons were recorded under conditions identical to those in control mice (i.e., Cs-intracellularly, 11 mM glucose, glutamate receptor blockers in ACSF). As in normoglycemic animals (8), neither sIPSC frequency (4.1 ± 1.7 Hz, control ACSF; 3.8 ± 1.3 Hz, insulin; n = 8; P = 0.55; Fig. 5) nor amplitude (37.5 ± 2.3 pA, control ACSF; 37.4 ± 3.3 pA,
insulin; n = 8; P = 0.93) were altered by insulin in DMV neurons from diabetic mice.

The effects on sIPSCs of forskolin and insulin were also examined in DMV neurons in diabetic mice. Forskolin significantly increased the frequency of sIPSCs in seven of eight cells (4.8 ± 1.5 Hz, control ACSF; 7.6 ± 1.7 Hz, forskolin; n = 8; P < 0.003; Fig. 6), with no change in amplitude (45.8 ± 8.0 pA, control ACSF; 48.0 ± 8.9 pA, forskolin; n = 8; P > 0.05). Forskolin increased sIPSC frequency in DMV neurons from diabetic mice, similar to normoglycemic mice.

The effect of insulin on sIPSC frequency and amplitude in the presence of forskolin was examined in 15 neurons from 5 diabetic mice. With the addition of insulin, there was no change in sIPSC frequency in the presence of forskolin in 10 of 15 neurons (two cells decreased, three increased; Kolmogorov–Smirnov test). Mean sIPSC frequency was 5.6 ± 1.1 Hz in forskolin and 6.1 ± 1.5 Hz with the addition of insulin (n = 15; P > 0.05; Fig. 7). Mean sIPSC amplitude was also unchanged (49.0 ± 5.2 pA, forskolin; 47.1 ± 5.7 pA, insulin; P > 0.05). To assess the effects of forskolin and insulin on action potential-independent inhibitory transmission in diabetic animals, DMV neurons were recorded in the presence of TTX and glutamate receptor antagonists. In diabetic mice, there was no change in mIPSC frequency (4.8 ± 1.2 Hz, forskolin; 4.5 ± 0.8 Hz, insulin; n = 11; P = 0.49; Fig. 7) or amplitude (38.3 ± 3.4 pA, forskolin; 35.8 ± 3.6 pA, forskolin + insulin; P > 0.05). Unlike results from normoglycemic mice, the addition of insulin in the continuous presence of forskolin failed to change sIPSC or mIPSC frequency in DMV neurons from diabetic mice.

DISCUSSION

Whereas insulin inhibits glutamate release, it does not affect GABA release in the DMV under normal conditions (8). This study showed that in the presence of forskolin, which presumably elevates cAMP in GABAergic terminals (11), insulin decreased inhibitory neurotransmission in the DMV in normoglycemic, but not hyperglycemic, mice. Application of the adenylate cyclase agonist forskolin [10 μM (11–13)] increased the frequency of IPSCs significantly. In the presence of forskolin, application of 1 μM insulin significantly reduced the frequency of IPSCs in DMV neurons from normoglycemic mice. The effect of insulin was blocked by preapplication of the Golgi-disrupting agent, brefeldin-A (5 μM), or the GLUT4 antagonist, indinavir (50 μM), suggesting that the cAMP-dependent insulin effect requires protein trafficking and perhaps GLUT4 activity. When an inactive analog of forskolin, 1–9-dideoxyforskolin, was used in place of forskolin, insulin had no effect on IPSC frequency or amplitude.

For these experiments, 11 mM glucose (equivalent to 198 mg/dl) was used for comparison to similar studies conducted in the brain stem, which widely utilize 10–11 mM glucose (4, 8, 11–13, 57, 58). Previous studies have shown that exposure of 4 h or more to lower glucose levels (i.e., 2.5 mM) significantly reduces activity of and may be damaging to cells in this region (6, 7, 21). The dorsal vagal complex is inundated with fenestrated capillaries (25), providing a substrate for large-molecule penetration, and the (minimal) blood-brain barrier in both NTS and DMV is permeable to circulating molecules, including glucose (38, 43). Thus, although glucose concentration has never been measured directly in the vagal complex, 11 mM glucose; n = 8; P = 0.93) were altered by insulin in DMV neurons from diabetic mice.

The effects on sIPSCs of forskolin and insulin were also examined in DMV neurons in diabetic mice. Forskolin significantly increased the frequency of sIPSCs in seven of eight cells (4.8 ± 1.5 Hz, control ACSF; 7.6 ± 1.7 Hz, forskolin; n = 8; P < 0.003; Fig. 6), with no change in amplitude (45.8 ± 8.0 pA, control ACSF; 48.0 ± 8.9 pA, forskolin; n = 8; P > 0.05). Forskolin increased sIPSC frequency in DMV neurons from diabetic mice, similar to normoglycemic mice.

The effect of insulin on sIPSC frequency and amplitude in the presence of forskolin was examined in 15 neurons from 5 diabetic mice. With the addition of insulin, there was no change in sIPSC frequency in the presence of forskolin in 10 of 15 neurons (two cells decreased, three increased; Kolmogorov–Smirnov test). Mean sIPSC frequency was 5.6 ± 1.1 Hz in forskolin and 6.1 ± 1.5 Hz with the addition of insulin (n = 15; P > 0.05; Fig. 7). Mean sIPSC amplitude was also unchanged (49.0 ± 5.2 pA, forskolin; 47.1 ± 5.7 pA, insulin; P > 0.05). To assess the effects of forskolin and insulin on action potential-independent inhibitory transmission in diabetic animals, DMV neurons were recorded in the presence of TTX and glutamate receptor antagonists. In diabetic mice, there was no change in mIPSC frequency (4.8 ± 1.2 Hz, forskolin; 4.5 ± 0.8 Hz, insulin; n = 11; P = 0.49; Fig. 7) or amplitude (38.3 ± 3.4 pA, forskolin; 35.8 ± 3.6 pA, forskolin + insulin; P > 0.05). Unlike results from normoglycemic mice, the addition of insulin in the continuous presence of forskolin failed to change sIPSC or mIPSC frequency in DMV neurons from diabetic mice.
glucose is within the normal range of blood glucose concentration, and this could reasonably approximate glucose concentration in this brain stem region. However, to identify potential effects of glucose concentration on the effect of insulin, we also examined the forskolin and insulin effects in 2.5 mM glucose, which yielded nearly identical results to those in 11 mM glucose. This indicates that any insulin-dependent glucose utilization in inhibitory synaptic terminals was effective over a relatively wide range of glucose concentration.

In the presence of forskolin, insulin reduced the frequency of both sIPSCs (75% of cells responded) and mIPSCs (69% of cells responded) similarly, and altered the PPr of evoked IPSCs in 56% of the cells tested. Additionally, the coefficient of variation analysis indicated that insulin effects in all five cells displaying a change in PPr (• in lower left quadrant of the graph) all lie under the diagonal line (r = π), indicating a presynaptic region of origin of the effect on PPr; • indicate cells that were unaffected by insulin. The black square (■) represents the means ± SE for the nine recorded cells. D: PPr plot for individual cells shows decrease in PPr after forskolin application in five cells and an increase in PPr with the addition of insulin. E: forskolin significantly decreased PPr vs. control ACSF (n = 5; †P < 0.009). The addition of insulin significantly increased the PPr in these neurons (n = 5; *P < 0.007).

Fig. 4. Insulin altered the coefficient of variation and decreased the evoked IPSC (eIPSC) paired-pulse ratio (PPr) in the presence of forskolin in DMV neurons from normoglycemic mice. A: overlapping, averaged traces showing eIPSCs recorded in a DMV neuron after paired electrical stimulation of the nucleus of the solitary tract (NTS) in control artificial cerebrospinal fluid (ACSF), forskolin (10 μM), and forskolin + insulin (1 μM). Averages of 20 responses are shown for each condition. B: averaged eIPSCs, normalized to the amplitude of the first response in A in control ACSF, forskolin and forskolin + insulin. C: plot of the ratio of the coefficient of variation of the first eIPSC amplitude in forskolin (CV₁forsk) over the CV₂ in forskolin + insulin (CV₁ins; r = CV₁forsk/CV₁ins) against the ratio of the eIPSC amplitude in forskolin + insulin (eIPSC ins) and in forskolin (eIPSC forsk; π = eIPSC ins/eIPSC forsk) for nine cells. The five cells that demonstrated a change in PPr (• in lower left quadrant of the graph) all lie under the diagonal line (r = π), indicating a presynaptic region of origin of the effect on PPr; • indicate cells that were unaffected by insulin. The black square (■) represents the means ± SE for the nine recorded cells. D: PPr plot for individual cells shows decrease in PPr after forskolin application in five cells and an increase in PPr with the addition of insulin. E: forskolin significantly decreased PPr vs. control ACSF (n = 5; †P < 0.009). The addition of insulin significantly increased the PPr in these neurons (n = 5; *P < 0.007).

Fig. 5. Insulin did not change sIPSC frequency in DMV cells from hyperglycemic mice. Representative traces from DMV cells in control ACSF (A) and insulin (1 μM) (B). C: cumulative probability plot showing that insulin did not alter sIPSC interevent interval in the neuron shown in A and B. D: insulin did not change mean sIPSC frequency in hyperglycemic mice (n = 8; P > 0.05).
elevated cAMP levels occurs at receptors located on the pre-
synaptic terminal of GABAergic neurons.

Because the DMV receives robust input from the NTS, these
results imply that cAMP-dependent insulin modulation of
GABAergic inhibition in the DVC is regulated by protein
translocation in the cells and/or terminals of NTS GABAergic
neurons. The present results concur with previous studies
demonstrating that effects of neuromodulators on synaptic
inhibition in the DMV often require elevated cAMP levels and
are due to cAMP-dependent receptor translocation (11–13). In
previous studies, translocation of peptide receptors to the
membrane was shown to be initiated by elevated cAMP in
synaptic terminals, which can occur subsequent to agonist
binding to $G_{\alpha}$ protein-coupled receptors or after visceral
deafferentation (11–15). As for cAMP-dependent responses to
other peptides, insulin might, thus, be expected to gate inhibi-
tion in a state-dependent fashion, such as in the presence of
substances that activate $G_{\alpha}$-coupled receptors or reduced va-
gal afferent activity, with cAMP elevation leading to insertion
of the insulin receptor and/or GLUT4 into the membrane.
Insulin effects were blocked by brefeldin-A, which prevents
protein trafficking to the membrane (11), consistent with this
conclusion. The protein trafficking effect of elevating cAMP in
inhibitory terminals is rapid (i.e., within ~5 min) and persists
for only about 60 min (14, 15). It is reasonable to assume that
insulin receptor or GLUT4 trafficking would be regulated
similarly.

In the context of gastrointestinal function and metabolism,
the influence of insulin in the vagal complex might be altered
by functional deafferentation that can occur after gastric bypass
surgery or by peptides (e.g., CCK) released from the gut
postprandially. A rapid and brief protein trafficking resulting in
a functional response to insulin may accommodate a periodic
need for responses, as occurs during feeding. Interestingly,
application of insulin essentially counteracted the cAMP-
induced increase in GABA release in the DMV, returning IPSC

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**Fig. 6.** Forskolin significantly increased sIPSC and mIPSC frequency in DMV neurons from hyperglycemic mice. Representative traces showing sIPSCs in a
DMV neuron from a hyperglycemic mouse recorded in control ACSF (A) and forskolin (10 μM) (B). C: cumulative probability plot showing a change in
interevent interval in the neuron shown in A and B. Forskolin application significantly increased sIPSC frequency in this neuron ($P < 0.05$). D: forskolin
significantly increased mean sIPSC ($n = 8$; $*P < 0.002$) and mIPSC ($n = 11$; $*P < 0.05$) frequency in DMV neurons from hyperglycemic mice.

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**Fig. 7.** Insulin applied in the presence of forskolin did not affect IPSC frequency in DMV neurons from hyperglycemic mice. Representative traces depicting
sIPSCs from a DMV neuron in the presence of forskolin (10 μM) (A) and forskolin + insulin (1 μM) (B). C: cumulative probability plot showing that sIPSC
interevent interval in the neuron shown in A and B was not altered by insulin applied in the presence of forskolin ($P > 0.05$). D: insulin did not change sIPSC
($n = 15$; $P > 0.05$) or mIPSC ($n = 11$; $P > 0.05$) frequency in the presence of forskolin in DMV neurons from hyperglycemic mice. E: pie chart showing
percentage of neurons that responded to insulin. Insulin decreased sIPSC frequency in only 2 of 15 neurons, and it decreased mIPSC frequency in only 3 of 11
cells.

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frequency to preforskolin levels. This implies a possible counterregulatory role for insulin on the effects of feeding peptides that elevate intraterminal cAMP. Much inhibitory synaptic input to the DMV is likely to arise from GABAergic NTS neurons, the majority of which receive viscerosensory input (17, 24), inferring a functional interaction between feeding-related peptides and insulin on central vago-vagal reflexes during a meal.

Insulin binds to the insulin receptor, which leads to cAMP-dependent phosphorylation of insulin receptor substrates (IRS), activation of downstream kinases (e.g., PKC), and ion channel modulation. In cells from several tissues (e.g., adipose, skeletal muscle), insulin receptor binding results in the translocation from internal stores to the membrane of a glucose transporter (GLUT4), where it functions to facilitate glucose transport into the cell. Eliminating or blocking any of the components in this pathway disrupts regulation of energy homeostasis (36, 44, 45). IRS2 levels can be stimulated by cAMP (29, 40). Thus, it is possible that the putatively low normal resting levels of cAMP in GABA terminals synapsing onto DMV neurons (11–13, 15) prevent insulin effects by obviating activation of the IRS pathway. Whereas other glucose transporters are located in the vagal complex, there is ample functional evidence for GLUT4 activity in vagal neurons (9, 55), which also contains insulin receptors. Here, inhibition of GLUT4 also prevented the insulin effect, implying a role for the transporter in cAMP-dependent insulin signaling in the DMV. Indinavir is a viral protease inhibitor, and nonantiviral effects that are independent of GLUT4 require long-term exposure (23, 30). Moreover, indinavir’s potency is 8- to 10-fold greater for GLUT4 than for GLUT2, and much greater than for other GLUTs (39), consistent with the hypothesis that GLUT4 participates in the response to insulin in the DMV. It seems likely that cAMP-dependent translocation of the insulin receptor and/or the glucose transporter to the terminal membrane is required for the effect of insulin on GABA release. It is impossible to tell from the present data which mechanism predominates, but the lack of cAMP-dependent insulin effect in the presence of a GLUT4 blocker or a protein-trafficking inhibitor suggests that translocation to the membrane of one or more elements in this pathway is likely.

In STZ-treated, diabetic mice, DMV neurons respond to forskolin with an increase in IPSC frequency, similar to effects in DMV neurons from normoglycemic control mice. Insulin did not, however, affect GABA release in DMV cells from diabetic mice after forskolin treatment, unlike in control animals. Because forskolin increased IPSC frequency in both normoglycemic and hyperglycemic mice, it seems unlikely that a disruption of cAMP function alone accounts for the inability of insulin to suppress inhibition in diabetic mice. Therefore, in hyperglycemic animals, there is likely an altered component of the insulin receptor cascade that interacts with the cAMP pathway. Neuronal GLUT4 is altered in diabetes, depending on brain region and diabetes model (2, 3, 16, 19, 33, 34, 54). Thus, one potential site of the dysfunction in diabetic mice is GLUT4 expression and/or translocation to the membrane. Loss of function in any of a number of other stages in the pathway, including insulin receptor translocation, IRS activation, or glucokinase activity could also contribute to the inability of insulin to alter GABA release, even in the presence of elevated cAMP. Understanding which point(s) in the pathway are dysregulated may be important for determining means of restoring vagal motor deficits associated with diabetes.

Several aspects of cellular function in the DVC are altered in diabetic mice, relative to their normoglycemic counterparts. Brief (21) or longer-term (8, 58) hyperglycemia alters behavior of neurons in the DVC to cause downstream effects on gastric motility and tone and on vagal motor neuron function. In STZ-treated diabetic mice that have been hyperglycemic for several days, the frequency of mEPSCs in the DMV is significantly increased vs. normoglycemic control mice (58). Insulin also induces TRPV1 receptor translocation to the synaptic terminal membrane in the DMV of STZ-treated diabetic mice, restoring the robust functional increase in glutamate release following TRPV1 activation seen in normal animals (18, 58). In control mice, insulin decreases sEPSC frequency and spontaneous action potential firing in gastric-related motor neurons of the DMV (8). Insulin applied in the hypothalamus or DVC reduces hepatic gluconeogenesis in rodents, and this effect is vagally mediated (22, 41, 42, 46). While insulin-induced hypoglycemia enhances vagal activity (26), hyperglycemia depresses vagal tone (37). The finding that cAMP-mediated, insulin-induced suppression of GABA release is abrogated in the DVC of diabetic mice represents further evidence to support the hypothesis that cellular function in vagal circuits is altered after several days of hyperglycemia/hypoinsulinemia in a manner that is sustained after standardization of ionic and glucose levels in slices. The functional role for this neuroplasticity is not known, but a loss of the potential disinhibitory effect of insulin might contribute to diabetes-associated gastroparesis, delayed gastric emptying, reduced gastric acid secretion and pancreatic polypeptide release, or increased hepatic gluconeogenesis (22, 27, 28, 31, 32, 49), especially if insulin normally regulates effects of feeding-related peptides on vagal reflexes. Vagal motor neuron activity is tightly regulated by synaptic inputs, especially arising from the NTS, and subtle dysregulation of this neural circuit can have profound effects on parasympathetic motor activity. Thus, the role of altered insulin effects on vagal motor neurons in regulating visceral function warrants further study.

Perspectives and Significance

Elevating cAMP levels with forskolin permits an effect of insulin on synaptic inhibition that is not seen under baseline conditions. This involves cAMP-dependent protein trafficking mechanisms and may require glucose transporter function. This cAMP-dependent insulin activity does not occur in diabetic animals, suggesting that there is a dysfunction in insulin receptor signaling that interacts with the cAMP signaling cascade in inhibitory neuron terminals that synapse onto DMV cells. Because GABA neurons of the NTS project heavily to the DMV and modulation of responses evoked from the NTS was evident with elevated cAMP, these neurons are a likely source of the inhibitory input involved in the effects seen in control mice, implying a role for the insulin effect in vagal reflex processing. Similar cAMP-dependent inhibitory mechanism has been shown previously in the DVC with other neurotransmitters, such as opioids, NPY, CCK, PPY, and 5-HT (11–13). Thus, the cAMP-dependent receptor trafficking may regulate how DMV neurons respond to input from other parts of the brain and the viscera postprandially, as well as to...
insulin-mediated endocrine influence. This mechanism is disrupted in diabetes, perhaps contributing to vagally mediated visceral dysregulation associated with the disease. Further investigation into the effects of diabetes on central vagal circuitry and the implication of these changes for metabolic regulation may prompt development of novel therapies for treating diabetes or its symptoms, based on regulation of parasympathetic neural function.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: C.B.B. and B.N.S. conception and design of research; C.B.B. 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. and B.N.S. edited and revised manuscript; C.B.B. and B.N.S. approved final version of manuscript.

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