Transient Receptor Potential Vanilloid Type 1–Dependent Regulation of Liver-Related Neurons in the Paraventricular Nucleus of the Hypothalamus Diminished in the Type 1 Diabetic Mouse

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The paraventricular nucleus (PVN) of the hypothalamus controls the autonomic neural output to the liver, thereby participating in the regulation of hepatic glucose production (HGP); nevertheless, mechanisms controlling the activity of liver-related PVN neurons are not known. Transient receptor potential vanilloid type 1 (TRPV1) is involved in glucose homeostasis and colocalizes with liver-related PVN neurons; however, the functional role of TRPV1 regarding liver-related PVN neurons has to be elucidated. A retrograde viral tracer was used to identify liver-related neurons within the brain-liver circuit in control, type 1 diabetic, and insulin-treated mice. Our data indicate that TRPV1 regulates liver-related PVN neurons. This TRPV1-dependent excitation diminished in type 1 diabetic mice. In vivo and in vitro insulin restored TRPV1 activity in a phosphatidylinositol 3-kinase/protein kinase C–dependent manner and stimulated TRPV1 receptor trafficking to the plasma membrane. There was no difference in total TRPV1 protein expression; however, increased phosphorylation of TRPV1 receptors was observed in type 1 diabetic mice. Our data demonstrate that TRPV1 plays a pivotal role in the regulation of liver-related PVN neurons. Moreover, TRPV1-dependent excitation of liver-related PVN neurons diminishes in type 1 diabetes, thus indicating that the brain-liver autonomic circuitry is altered in type 1 diabetes and may contribute to the autonomic dysfunction of HGP. *Diabetes* **61:1381–1390, 2012**

The central nervous system (CNS) plays a critical role in the regulation of glucose metabolism and energy homeostasis via the activity of neurons controlling the autonomic nervous system (1–4). The paraventricular nucleus (PVN) of the hypothalamus incorporates signals from many different brain areas, including a variety of hypothalamic nuclei involved in the maintenance of energy and glucose homeostasis (e.g., arcuate nucleus, dorsomedial hypothalamus, etc.) (5–8) and regulates the sympathetic (SNS) and parasympathetic (PNS) nervous system, controlling visceral functions, including hepatic glucose production (HGP). Lesion and chemical stimulation studies revealed direct PVN involvement in plasma glucose control (9,10), indicating that the activity of preautonomic PVN neurons plays a pivotal role in the regulation of HGP. Moreover, blockade of γ-aminobutyric acid_A receptors in the PVN caused a pronounced increase in plasma glucose concentration via sympathetic nerves to the liver (11). Increased SNS activity or decreased PNS activity to the liver leads to enhanced hepatic gluconeogenesis (12–14) and thereby to increased HGP. Increased HGP is found in both type 1 and type 2 diabetic patients (15,16), indicating at least partially dysregulated central autonomic control via the SNS and PNS (14). However, the mechanisms regulating the activity of liver-related preautonomic PVN neurons have remained elusive until now.

Transient receptor potential vanilloid type 1 (TRPV1) is a ligand-gated nonselective cation channel with high Ca^{2+} permeability (17). Activation of peripheral TRPV1 receptors impacts glucose homeostasis (18), and although the role of peripheral TRPV1 regarding diabetes has been well studied (19–21) its central role related to glucose homeostasis has not been determined. TRPV1 is widely expressed in the CNS, including the critical nuclei involved in central regulation of glucose homeostasis, such as the PVN (22–24). Moreover, TRPV1 receptors colocalize with liver-related PVN neurons (24). Activation of TRPV1 receptors enhances the release of neurotransmitters (25–27); therefore, TRPV1 also may play a critical role in the regulation of liver-related preautonomic PVN neurons, thus controlling the autonomic output to the liver. In addition, insulin and insulin-like factor-1 (IGF-1) enhance TRPV1-mediated currents, suggesting that an insulin deficiency might contribute to downregulation of TRPV1-dependent control of liver-related PVN neurons and to neuronal complications associated with diabetes.

In this study, we combined retrograde, transneuronal viral labeling (PRV-152) to identify liver-related preautonomic neurons in the PVN with whole-cell patch-clamp electrophysiology to determine the TRPV1-dependent regulation of liver-related PVN neurons in control and type 1 diabetic conditions. Moreover, we tested the hypothesis that TRPV1-dependent excitation of liver-related PVN neurons is downregulated in a mouse model of type 1 diabetes. Our data demonstrate that TRPV1-dependent regulation of liver-related PVN neurons diminishes in type 1 diabetes, and insulin has the ability to control TRPV1 activity.

RESEARCH DESIGN AND METHODS

Experiments were performed on male CD1 mice (7–8 weeks old; Harlan) following the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by Tulane University's Institutional Animal Care and Use Committee.

Injection of PRV-152. A retrogradely transported viral vector strain isogenic with PRV Bartha that reports enhanced green fluorescent protein (EGFP), known as PRV-152 (supplied by Dr. L.W. Enquist), was used to identify liver-related PVN neurons (24,28). Under anesthesia, a small transverse incision was made to expose the liver for injection. Three injections (2 μL each) of PRV-152 at a titer of 1×10^8 plaque-forming units (pfu)/mL were made into the median

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lobe of the liver. Each injection site was immediately sealed with one drop of liquid bandage to prevent virus leakage (24). The animals were maintained in a biosafety level 2 facility up to 96–110 h postinjection.

Streptozotocin injection and insulin replacement. The mice were kept fasting overnight (10–14 h) and then injected intraperitoneally with streptozotocin (STZ) (200 mg/kg; Alexis Biochemicals) dissolved in 0.1 mol/L citrate buffer (pH 4.5). Control animals received citrate buffer injections only. Body weight and blood glucose levels (OneTouch Ultra) were monitored prior to injection and daily afterward. Mice having blood glucose levels >300 mg/dL (16.6 mmol/L) for at least 3 days were considered hyperglycemic and used for additional experiments. After the onset of hyperglycemia, the animals were injected with PRV-152. A group of type 1 diabetic mice received a sustained-release insulin implant inserted subcutaneously (LinBit; LinShin, Toronto, Ontario, Canada) and were then injected with PRV-152.

Whole-cell patch-clamp recordings. Brain slices were prepared as previously described. Whole-cell patch-clamp recordings were performed as previously described in detail (4,25,29). Excitatory postsynaptic currents (EPSCs) were examined at a holding potential of −60 mV. Electrophysiological signals were recorded using an Axoclamp 700B amplifier (Molecular Devices) and stored in a computer using a Digidata 1440A digitizer and pClamp 10 software (Molecular Devices). Synaptic currents were analyzed offline using MiniAnalysis (Synaptosoft).

Drug application. Recordings were performed with tetrodotoxin (1 μmol/L; Tocris Bioscience) in artificial cerebrospinal fluid (aCSF) to block action potentials and monitor miniature (m)EPSCs. The TRPV1 agonist capsaicin (1 μmol/L; Tocris Bioscience), the TRPV1 antagonist 5′-iodoresiniferatoxin (5′-iRFT) (1 μmol/L; Tocris Bioscience), and the Golgi-disrupting agent brefeldin A (5 μmol/L; Sigma) were dissolved in ethanol and diluted in aCSF (final concentration of ethanol <0.01% by volume). A protein kinase C (PKC) activator phorbol 12-myristate 13-acetate (PMA) (3 μmol/L; Tocris Bioscience), a PKC inhibitor GF 109203× (bisindolylmaleimide [BIM], 2 μmol/L; Tocris Bioscience), and a phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin (3 μmol/L; Sigma) were dissolved in DMSO and diluted in aCSF (final DMSO concentration <0.01%). Insulin (1 μmol/L Novolin R; Novo Nordisk) was diluted in aCSF.

Data analysis. The effects of agonists and antagonists on mEPSC frequency and amplitude were analyzed within individual cells using the Kolmogorov-Smirnov test. The effects of agonists and antagonists across neuron groups were analyzed using a paired two-tailed Student *t* test or between different groups using one-way ANOVA ($P < 0.05$). Values are expressed as means ± SEM.

Western blot detection and quantification. Sections were made from control and type 1 diabetic mice, and the PVN was microdissected, homogenized in lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% SDS, 0.1% Na-deoxycholate, and 1% NP-40; 1 mmol/L Na_3VO_4; and 1:400 vol/vol protease inhibitor cocktail), and centrifuged (14,000 rpm, 4°C, 30 min). Aliquots containing 90 μg of protein (quantified by a Micro BCA Protein Assay kit; Pierce) were separated by 4–12% Bis-Tris gels (Novex), transferred to polyvinylidene fluoride (Millipore), and incubated in Odyssey blocking buffer and with anti–phospho-TRPV1 (0.25 μg/mL; Cosmo Bio) and anti-TRPV1 (1:500; Neuromics) antibody (4°C, overnight). Incubation was followed with goat anti-rabbit antibody (1:15,000; Li-Cor) labeled with infrared dye 800 in blocking buffer. Protein bands were detected by an Odyssey Infrared Imaging System (Li-Cor). Equal protein loading was confirmed by anti–β-actin antibody (1:5,000; Abcam). Western blot data were analyzed by one-way ANOVA ($P < 0.05$).

RESULTS

Injection of STZ resulted in hyperglycemia (487 ± 14 mg/dL; n = 42). The in vivo insulin treatment significantly decreased the blood glucose levels of STZ-induced diabetic mice (166 ± 39 mg/dL; n = 12). PRV-152 was used to identify liver-related neurons in the PVN of control, type 1 diabetic, and insulin-treated mice. At 96–110 h post-inoculation, we observed EGFP labeling indicating liver-related neurons in the PVN (Fig. 1*A*) consistent with previously published results in mice (24). Recordings were made from liver-related and non–liver-related PVN neurons.

TRPV1-dependent excitation of liver-related PVN neurons diminishes in type 1 diabetic animals. To investigate excitatory synaptic regulation of liver-related PVN neurons by TRPV1, we used whole-cell patch-clamp recordings. The frequency and amplitude of mEPSCs of liver-related PVN neurons were examined in the presence of tetrodotoxin (1 μmol/L) in slices from control and type 1 diabetic mice. To analyze the effects of hyperglycemia and insulin deficiency on TRPV1-dependent regulation of liver-related PVN neurons, capsaicin, a TRPV1 agonist, was applied while recording mEPSCs. Bath application of capsaicin (1 μmol/L) significantly increased the overall frequency of mEPSCs in liver-related PVN neurons from control mice (Fig. 2). The effect of capsaicin on the overall population was observed within 2 min of the drug reaching the slice and was maximal by 6 min. Under control conditions, the average mEPSC frequency was 2.58 ± 0.4 Hz (range 0.4–7.3). After application of capsaicin, the mean frequency of mEPSCs increased to 3.56 ± 0.67 Hz (0.5–10.4; n = 15; $P < 0.05$) (Fig. 2). Additional analysis of individual cells with the Kolmogorov-Smirnov test revealed that 6 of 15 cells had no significant increase in mEPSC frequency, indicating that not all liver-related PVN neurons have the same TRPV1-dependent regulation. Table 1 indicates the number and frequency response to capsaicin. There was no significant change in mEPSC amplitude. The average amplitude in neurons from control animals was 13.5 ± 0.8 pA (range 9.2–19.7) and 12.38 ± 0.4 pA (9.2–16.8; $P > 0.05$) after application of capsaicin.

In contrast, capsaicin did not increase the frequency of mEPSCs in liver-related PVN neurons from type 1 diabetic animals (Fig. 2). Under control conditions, the mean frequency was 1.89 ± 0.6 Hz (range 0.3–5.5; n = 8). After application of capsaicin, the frequency of mEPSCs was unchanged at 1.97 ± 0.63 Hz (0.4–5.6; n = 8; $P > 0.05$) (Fig. 2). The amplitude of mEPSCs in neurons from type 1 diabetic mice was 12.7 ± 1.25 pA (range 8.8–19) and 12.1 ± 1.14 pA (8.0–16.2; $P > 0.05$) after application of capsaicin. The normalized increase of mEPSC frequency in liver-related PVN neurons of control mice was 35.4 ± 6% (n = 15), whereas it was 3.1 ± 3.9% in type 1 diabetic mice (n = 8) (Fig. 2*I* and Table 1).

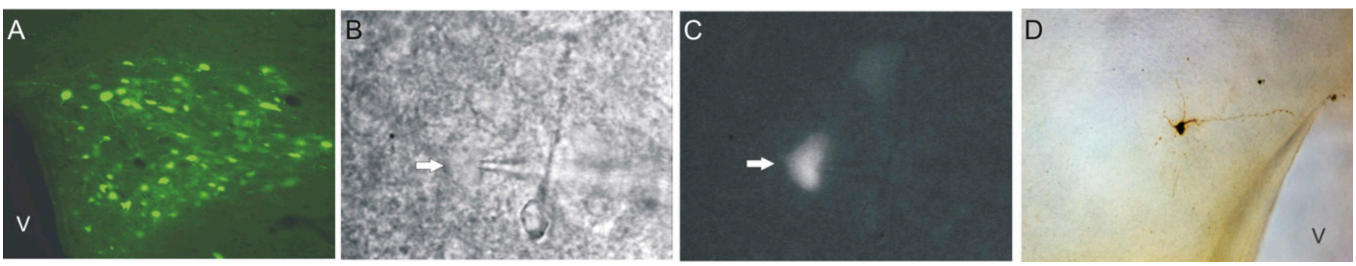

FIG. 1. Visualization of liver-related preautonomic PVN neurons. *A*: Hypothalamic section with EGFP-labeled liver-related preautonomic neurons 110 h after inoculation of the liver with PRV-152. *B*: Differential interference contrast image of an identified PVN neuron during recording. *C*: The same neuron under fluorescent light. *D*: The same liver-related PVN neuron shown in *B* and *C* after fixation and reaction to visualization with diaminobenzidine. Arrow points to the recorded cell. V, third ventricle. (A high-quality digital representation of this figure is available in the online issue.)

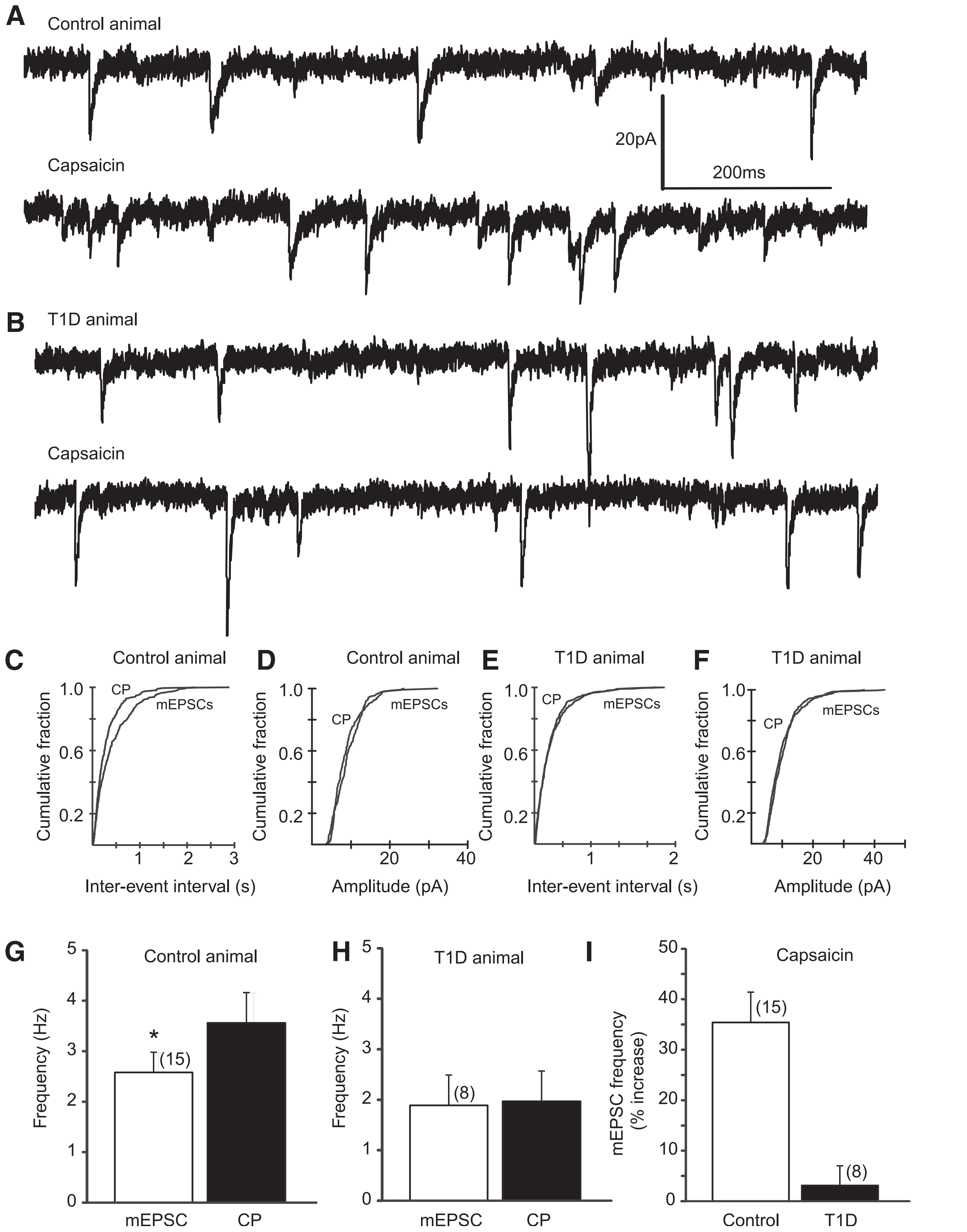

FIG. 2. TRPV1-dependent excitation of liver-related PVN neurons diminished in type 1 diabetic (T1D) mice. *A*: Application of capsaicin increases mEPSC frequency in control mice. Continuous recording of mEPSCs from control mice before (*upper trace*) and after (*lower trace*) capsaicin application. *B*: TRPV1-dependent increase of the frequency of mEPSCs diminished in T1D mice. Continuous recording of mEPSCs from T1D mice before (*upper trace*) and after (*lower trace*) capsaicin application. *C–F*: Cumulative event probability plots of interevent interval distribution in recordings from control (*C*) and T1D (*E*) mice and amplitude in control (*D*) and T1D (*F*) mice. *G–I*: Combined data showing the effect of capsaicin in control and T1D mice. Capsaicin increased mEPSC frequency in liver-related PVN neurons of control mice (*G*) but did not alter the mEPSC frequency of liver-related PVN neurons of T1D mice (*H*). *Significance (P < 0.05). *I*: Mean group changes showing the effect of capsaicin on the mEPSC frequency of liver-related PVN neurons in control and T1D mice.

TABLE 1
Frequency response to TRPV1 activation in liver-related and non–liver-related PVN neurons of control and type 1 diabetic mice

Animal/cell type	n cells	Frequency before capsaicin (Hz)	Frequency after capsaicin (Hz)	Range of frequency before capsaicin (Hz)	Range of frequency after capsaicin (Hz)	Normalized change (%)
Control						
Non–liver related						
All cell	13	2.29 ± 0.5	2.04 ± 0.4	0.5–7.3	0.3–4.9	−5 ± 6
Liver related						
All cell	15	2.58 ± 0.4	3.56 ± 0.6*	0.4–7.3	0.5–10.4	35.4 ± 6*
Increase based on Kolmogorov-Smirnov test	9	3.19 ± 0.6	4.7 ± 0.8*	1.5–7.3	2.2–10.4	49.6 ± 6*
No increase based on Kolmogorov-Smirnov test	6	1.67 ± 0.6	1.8 ± 0.6	0.4–4.7	0.5–5.0	14.0 ± 4
Type 1 diabetic						
Liver related						
All cell	8	1.89 ± 0.6	1.97 ± 0.6	0.3–5.5	0.4–5.6	3.1 ± 3.9
Insulin in the bath	8	1.7 ± 0.3	2.7 ± 0.3*	0.5–2.6	0.5–3.9	53.8 ± 22*
Systemic insulin	11	1.6 ± 0.2	2.2 ± 0.3*	0.4–2.8	0.3–4.1	37.0 ± 9*
Non–liver related						
Systemic insulin	8	2.3 ± 0.4	2.6 ± 0.6	0.7–4.2	0.4–5.6	8 ± 10

*Significance ($P < 0.05$).

To verify that the increased mEPSC frequency observed in control animals is attributed to TRPV1 receptor activation, 5′-iRFT (1 μmol/L), a TRPV1 receptor antagonist, was applied before and during application of capsaicin. 5′-iRFT alone did not result in a significant change in the frequency of mEPSCs (1.98 ± 0.26 Hz) in liver-related PVN neurons. Application of capsaicin in the presence of 5′-iRFT did not increase mEPSC frequency (1.76 ± 0.13 Hz; n = 7; $P > 0.05$), indicating that the effect of capsaicin is attributed to TRPV1 receptor activation.

To determine that the capsaicin response is specific to liver-related PVN neurons, recordings were conducted from non–liver-related PVN neurons. Under control conditions, the mean frequency was 2.29 ± 0.5 Hz (range 0.5–7.31; n = 13). Application of capsaicin did not alter the overall frequency of mEPSCs (2.04 ± 0.4 [0.3–4.9]; $P > 0.05$) (Table 1), indicating that the recorded non–liver-related PVN neurons did not respond to capsaicin.

Insulin restores TRPV1-dependent increase of mEPSC frequency in liver-related PVN neurons of type 1 diabetic mice. Slices from type 1 diabetic and control mice were incubated in insulin (1 μmol/L; ~30 min) to determine the effect on TRPV1-dependent excitation of liver-related PVN neurons. There was no significant effect from insulin alone on basal mEPSC frequency in control (2.5 ± 0.4 Hz, n = 15 vs. 1.9 ± 0.67 Hz, n = 7) or type 1 diabetic (1.89 ± 0.6 Hz, n = 8 vs. 1.71 ± 0.29 Hz, n = 8; $P > 0.05$) mice. Simultaneous application of capsaicin and insulin increased mEPSC frequency of liver-related PVN neurons of control mice from 1.9 ± 0.6 Hz (range 0.5–5.7) to 2.56 ± 0.7 Hz (0.6–6.4; $P < 0.05$). The magnitude of the increase was not different in the presence or absence of insulin in control mice (35.4 ± 6 vs. 41 ± 10%).

However, preincubation with insulin restored the effect of capsaicin on mEPSC frequency in liver-related PVN neurons of type 1 diabetic animals (Fig. 3). The average mEPSC frequency of liver-related PVN neurons from type 1 diabetic mice exposed to insulin was 1.71 ± 0.29 Hz (range 0.5–2.6; n = 8). Capsaicin in the presence of insulin significantly increased mEPSC frequency to 2.71 ± 0.36 Hz (0.5–3.9; $P < 0.05$) (Fig. 3*B*). The magnitude of the capsaicin-induced increase was greater than in neurons from type 1 diabetic animals without insulin incubation ($P < 0.05$) (Fig. 3*D*) and was similar to the capsaicin-induced increase seen in control mice ($P > 0.05$). These data indicate that acute exposure to insulin can restore the TRPV1-dependent regulation of liver-related preautonomic PVN neurons in type 1 diabetic mice.

Moreover, recordings were made from liver-related and non–liver-related PVN neurons of insulin-treated type 1 diabetic mice. The in vivo insulin treatment rescued the TRPV1-dependent regulation of liver-related PVN neurons. The average mEPSC frequency increased from 1.6 ± 0.2 Hz (range 0.4–2.8) to 2.2 ± 0.3 Hz (0.3–4.1; n = 11; $P < 0.05$) after capsaicin application (Fig. 3*C*). Nevertheless, capsaicin failed to increase mEPSC frequency in non–liver-related PVN neurons. The average frequency was 2.3 ± 0.46 Hz (0.7–4.2) and 2.6 ± 0.6 Hz (0.4–5.6; n = 8; $P > 0.05$) (Table 1).

Insulin modulates TRPV1 activity in a PI3K/PKC-dependent manner

PKC inhibition. To determine whether insulin acts via a PKC-dependent mechanism, the effect of a selective and potent PKC inhibitor, BIM, was tested. Slices were preincubated with insulin (1 μmol/L) and BIM (2 μmol/L) for ~30 min, and mEPSCs were recorded from liver-related PVN neurons of type 1 diabetic mice prior to and after capsaicin (1 μmol/L) application. The average frequency before capsaicin application was 1.5 ± 0.32 Hz (range 0.3–2.9; n = 7). Application of capsaicin failed to increase the frequency of mEPSCs in the presence of PKC inhibitor and insulin (1.26 ± 0.22 [0.5–1.97]; $P > 0.05$) (Fig. 4*A*).

PKC activation. To further verify the involvement of PKC, we preincubated the slices from type 1 diabetic animals with a PKC activator, PMA (3 μmol/L; ~30 min). Similar to the effect of insulin, preincubation of the slices with PMA restored the effect of capsaicin (Fig. 4*B*). In the presence of PMA, the average mEPSC frequency of liver-related preautonomic PVN neurons was 7.7 ± 2.3 Hz (range 2.1–16.2) and increased to 9.38 ± 2.54 Hz (3.3–18.6) after application of capsaicin (n = 6; $P < 0.05$). The average frequency of mEPSCs in the presence of PMA alone

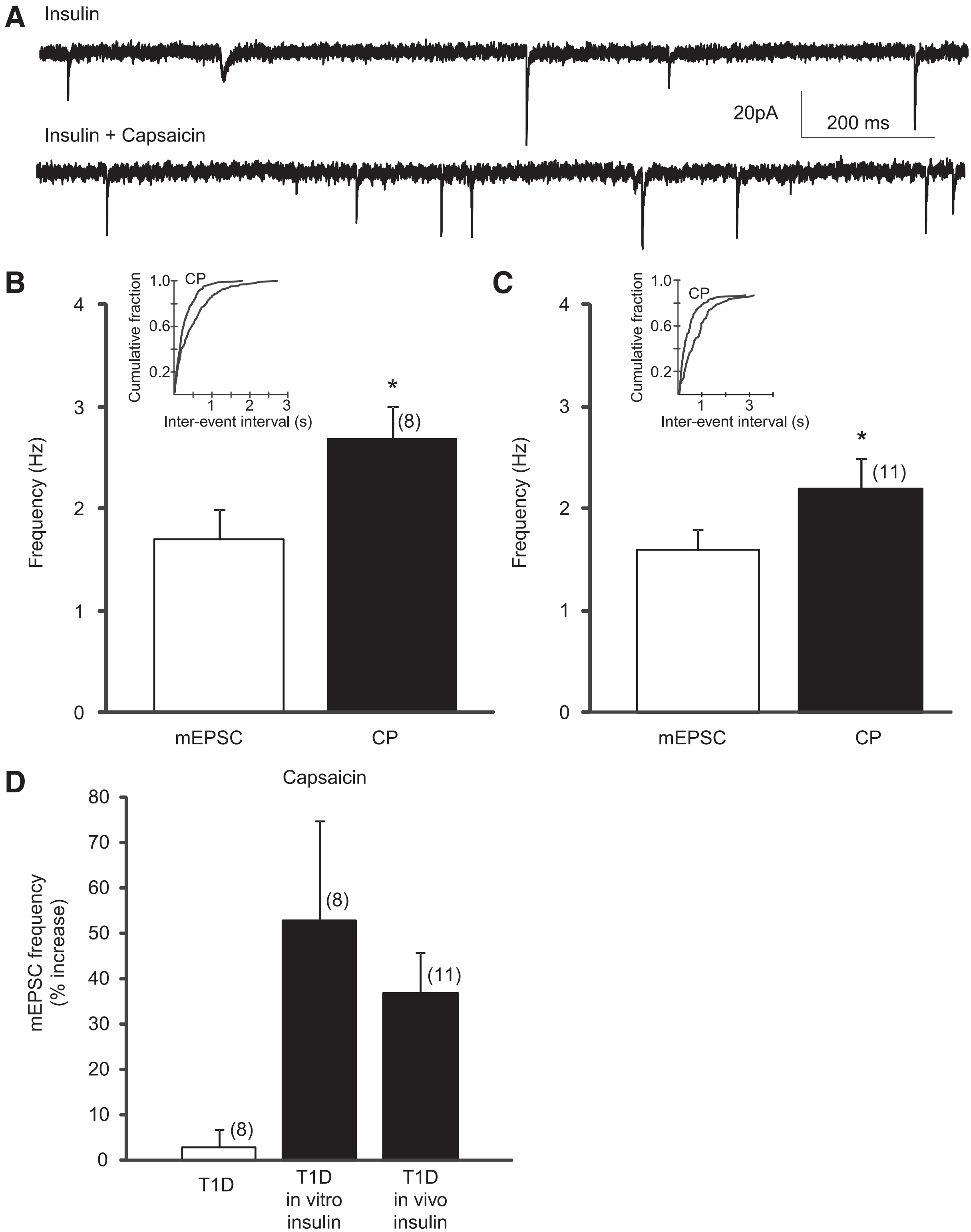

FIG. 3. TRPV1-dependent excitation of liver-related PVN neurons was restored in type 1 diabetic (T1D) mice with insulin. *A*: Continuous recording of mEPSCs from liver-related PVN neurons in the presence of insulin before (*upper trace*) and after (*lower trace*) administration of capsaicin. *B*: Combined data showing the increase of the frequency of mEPSCs in the presence of insulin. *Inset*: Cumulative event probability plot of the interevent distribution in the presence of insulin. *C*: Combined data showing the increase of the frequency of mEPSCs in T1D mice treated with insulin in vivo. *Inset*: Cumulative event probability plot of the interevent distribution in the presence of insulin. *Significance ($P < 0.05$). *D*: Mean group changes showing the effect of capsaicin administration on the frequency of mEPSCs of liver-related PVN neurons in T1D mice in the presence and absence of insulin.

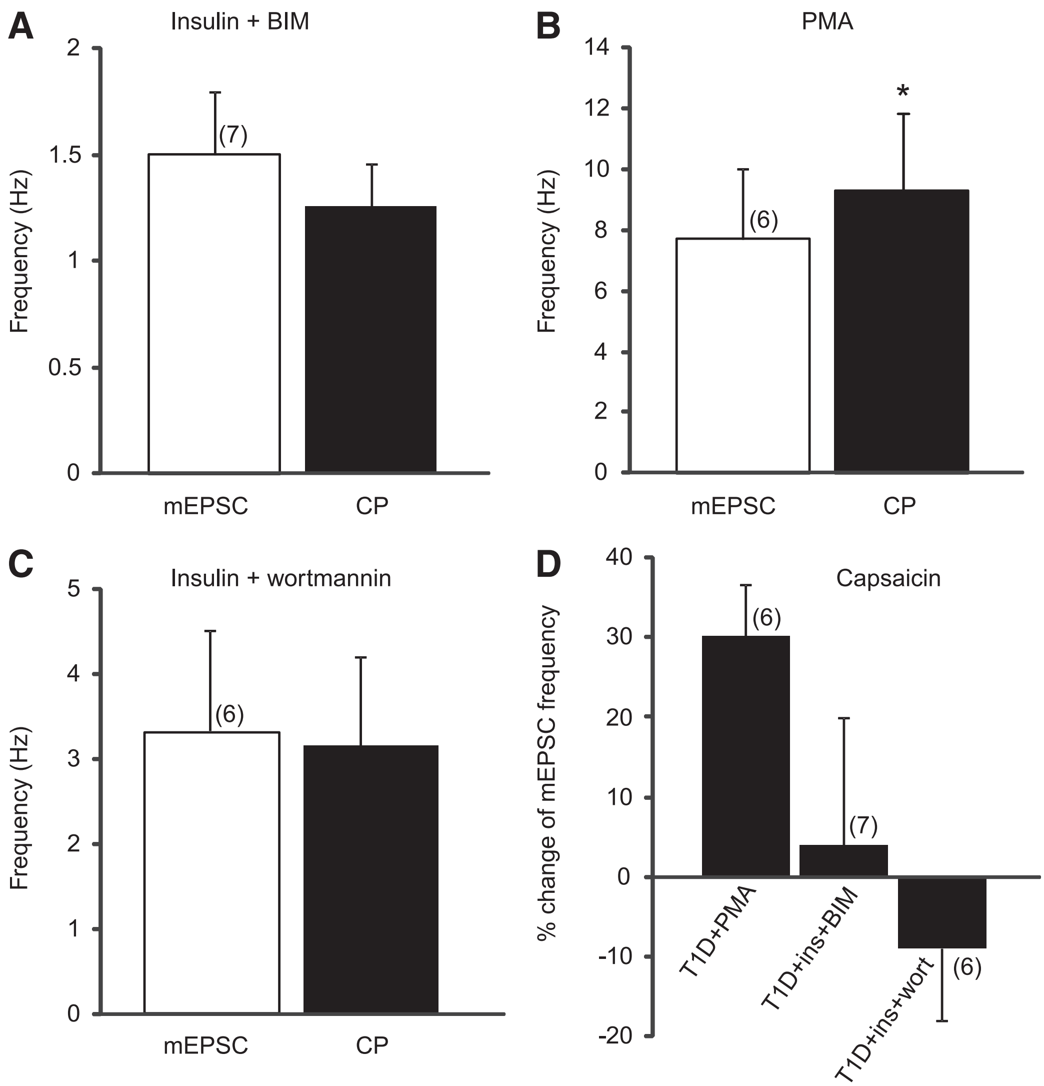

FIG. 4. Insulin modulates the TRPV1-dependent regulation of liver-related PVN neurons in a PI3K/PKC-dependent manner. *A*: A PKC inhibitor, BIM, prevented the insulin-caused reinstatement of TRPV1 activation in type 1 diabetic (T1D) mice. *B*: Activation of PKC with PMA mimicked the insulin-caused reinstatement of TRPV1-dependent increase in T1D mice. *$P < 0.05$. *C*: Wortmannin, a PI3K inhibitor, prevented the insulin-dependent reinstatement of effect in T1D mice. *D*: Mean group changes of normalized frequency showing PI3K/PKC-dependent modulation of TRPV1 receptors in T1D mice.

(7.7 ± 2.3 Hz) was significantly higher when compared with liver-related PVN neurons from control (2.58 ± 0.4 Hz) or type 1 diabetic (1.89 ± 0.6 Hz) mice. However, the magnitude of increase after capsaicin application (30.1 ± 6.5%) was similar to that observed in control animals (35.4 ± 6%).

The PI3K pathway. The involvement of PI3K in restoration of TRPV1 effect was examined using wortmannin, a PI3K inhibitor. Preincubation of slices with wortmannin (3 μmol/L) plus insulin (1 μmol/L) diminished the effect of capsaicin (1 μmol/L) compared with insulin alone in liver-related PVN neurons. In the presence of wortmannin and insulin, the average frequency was 3.3 ± 1.18 Hz (range 0.9–7.7) and 3.15 ± 1.07 Hz (0.5–6.5) after application of capsaicin ($n = 6$; $P > 0.05$) (Fig. 4*C*).

Phospho-TRPV1–to–TRPV1 ratio is higher in the PVN of type 1 diabetic mice. Because TRPV1-dependent excitation of liver-related PVN neurons is diminished in type 1 diabetic mice, the relationship between altered TRPV1 receptor activity and changes in receptor protein expression in the PVN of the hypothalamus was investigated by Western blot analysis. No significant difference in TRPV1 total protein expression was detected between the two groups ($n = 8$; $P > 0.05$) (Fig. 5), thus suggesting that the altered response to TRPV1 activation was not a result of diminished TRPV1 expression in type 1 diabetic mice. To explore the possible changes in phosphorylation of TRPV1 receptors, the phospho-TRPV1–to–TRPV1 ratio was analyzed. We found that the phospho-TRPV1–to–TRPV1 ratio was higher in the PVN of type 1 diabetic mice ($P < 0.05$) (Fig. 5).

Insulin-dependent TRPV1 receptor translocation. Insulin through PKC activation can reinstate TRPV1 function by recruiting receptors to the plasma membrane (30,31); therefore, we tested the insulin-dependent translocation of TRPV1. Brefeldin A was applied to disrupt the Golgi apparatus and interfere with receptor trafficking. The average mEPSC frequency in the presence of brefeldin A (5 μmol/L) plus insulin (1 μmol/L) was 2.73 ± 1.01 Hz (range 0.9–6.8) and 3.42 ± 1.39 Hz (0.9–9.6) after application of capsaicin (1 μmol/L; $n = 6$; $P > 0.05$) (Fig. 5), an increasing trend but not reaching significance.

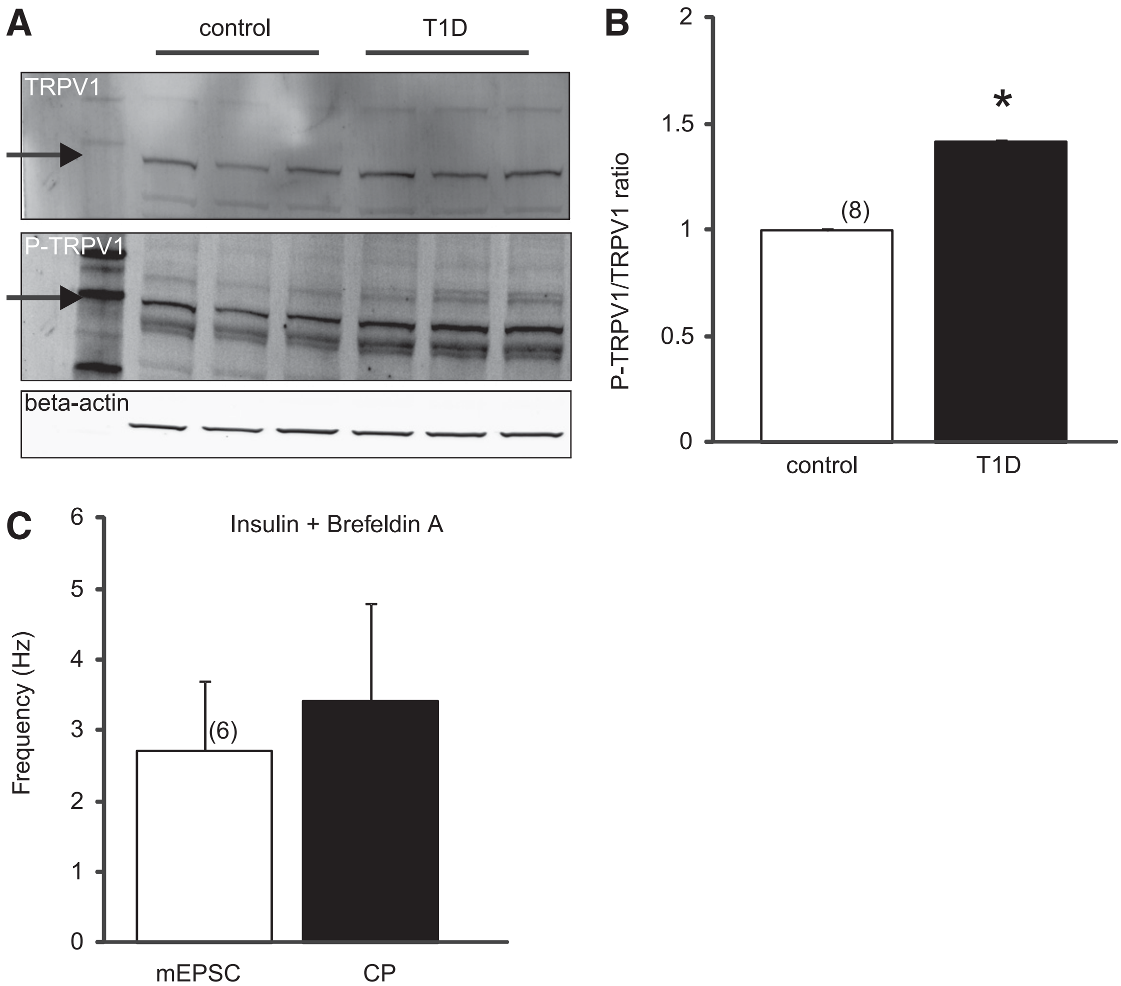

FIG. 5. TRPV1 expression levels were not different in STZ-induced diabetic mice; however, the phospho-TRPV1–to–TRPV1 ratio was higher in the PVN of type 1 diabetic (T1D) mice. *A*: Western blot showing total TRPV1 expression in the PVN from control and T1D mice (*upper panel*). Phospho-TRPV1 expression in the PVN of control and T1D mice (*middle panel*). Actin was used as a loading control (*lower panel*). *B*: Western blot densitometry analysis detected an increase of the phospho-TRPV1–to–TRPV1 ratio between control and T1D mice. *Significance (P < 0.05). *C*: Brefeldin A, which disrupts Golgi function and receptor trafficking, prevented the restorative effect of insulin on TRPV1 function.

DISCUSSION

This study demonstrates diminished TRPV1-dependent regulation of liver-related PVN neurons in a model of type 1 diabetes. The following novel findings emerged: *1*) TRPV1 receptors regulate liver-related PVN neurons; *2*) TRPV1-dependent excitation of liver-related preautonomic PVN neurons diminished in type 1 diabetic mice; *3*) in vivo and in vitro insulin restored the TRPV1-dependent regulation in type 1 diabetic mice; *4*) insulin controls TRPV1 receptor activity in a PI3K/PKC-dependent manner; *5*) insulin causes translocation of TRPV1; and *6*) increased phospho-TRPV1–to–TRPV1 ratio was observed in type 1 diabetic mice. The pivotal demonstration of diminished TRPV1-dependent regulation of liver-related PVN neurons in type 1 diabetic mice suggests altered central autonomic circuitry, possibly contributing to elevated HGP by decreasing the excitability of liver-related preautonomic PVN neurons. Our data support a regulatory role of central TRPV1 in the maintenance of glucose homeostasis; thus, modulation of TRPV1 in the PVN might represent a new approach to restoring adequate neural regulation of HGP.

Technical considerations. Using PRV-152, we labeled a neuronal population with direct and indirect connections to the liver (24,28,32). The spread of PRV-152 is strictly retrograde (33); therefore, the EGFP-labeling indicates liver-related PVN neurons. However, recently a subpopulation of neurons projecting both to the liver and epididymal white fat was identified in the hypothalamus and brainstem of mice (32). Therefore, it is possible that some of the PRV-labeled preautonomic neurons are "command neurons" projecting to other metabolically important organs not only to the liver. Nevertheless, to date, this PRV approach provides a unique opportunity to study liver-related PVN neurons and determine their synaptic properties, an elusive goal before now. Liver-related neurons become visible in other hypothalamic areas (e.g., arcuate nucleus, ventromedial nucleus of the hypothalamus, dorsomedial nucleus of the hypothalamus) ~6 days after PRV inoculation of the liver (32). These areas are very important for insulin signaling and HGP via the SNS and PNS (5,14,34) and may influence the activity of PVN neurons; thus, there exists the possibility that similar alteration in their neuronal activity can occur, further modulating preautonomic PVN neurons. However, to establish TRPV1-dependent regulation in other hypothalamic areas will require additional investigation and could be the subject of future studies.

Despite the specific identification of liver-related PVN neurons, this method does not allow for distinguishing between presympathetic and preparasympathetic liver-related PVN neurons. Moreover, we have observed that mEPSC frequency in some of the liver-related PVN neurons of control mice did not increase after capsaicin application, indicating that there might be a difference in the regulation of presympathetic and preparasympathetic neurons. This is supported by the observation that the majority (70%), but not all, of liver-related PVN neurons express TRPV1 receptors in the PVN (24). However, with the current approach, we cannot differentiate between presympathetic and preparasympathetic liver-related PVN neurons; this requires further investigation.

The harmful effects of PRV on neuronal properties have been extensively researched and addressed in several articles (35–37). There are no changes in the electrical properties of PRV-152–infected cells 18 h into the 24-h infection cycle (38). Numerous publications using PRV-152 to identify organ-specific neurons reported that the infected neurons have spontaneous synaptic inputs, the membrane properties of infected and uninfected cells were identical, and the virus did not have adverse effects on electrical properties (29,37,39). Our recordings were conducted ~96–110 h after the inoculation of the liver, an early time point for labeling liver-related PVN neurons without EGFP labeling outside of the PVN (32). None of the electrical properties were different from the uninfected cells. Although it remains possible that PRV-152 might alter some properties of the labeled neurons later, our recordings were carefully designed to minimize the likelihood of effects of long-term infection.

In our study, STZ was used to induce type 1 diabetes in CD1 male mice. Administration of STZ also could directly affect renal, hepatic, and muscle tissues, as well as neurons (40); however, the direct effects of STZ in PVN neurons are likely negligible because we examined the TRPV1-dependent responses 6–7 days after hyperglycemia, and there is no measurable STZ 2 h after injection (41). Although it is possible that STZ triggers unappreciated events, the drug by itself is unlikely to have a direct effect on the recordings.

TRPV1-dependent regulation of liver-related PVN neurons. HGP can be stimulated by hormonal (e.g., increased glucagon release) and neural (e.g., increased activity of sympathetic nerves or decreased activity of parasympathetic input to the liver) signals (42,43). However, our understanding of the brain networks regulating the activity of the SNS and PNS is not well established, especially regarding mechanisms controlling the activity of liver-related PVN neurons. In vivo studies indicate the direct involvement of the PVN in glucose control (9,10), suggesting that the activity of preautonomic PVN neurons has a pivotal role in governing HGP. Furthermore, a recent study showed that central administration of anandamide, also activating TRPV1, regulates HGP and systemic lipolysis (3), further indicating the importance of the central neuronal circuitry in the regulation of glucose metabolism. Our data demonstrating that there is a TRPV1-dependent control of liver-related PVN neurons provide novel information about the synaptic control of autonomic neural networks. Of interest, application of capsaicin did not alter the overall mEPSC frequency of non–liver-related PVN neurons. Analysis of each individual cell with the Kolmogorov-Smirnov test revealed that capsaicin increased mEPSC frequency only in one cell out of all recorded. This neuron could be a liver-related neuron not infected at this stage (32) or another preautonomic PVN neuron. Moreover, the absence of TRPV1-dependent control of non–liver-related PVN neurons indicates that at the level of the PVN, TRPV1 might play a significant role in the regulation of the synaptic activity of preautonomic, liver-related PVN neurons and ultimately in neural control of HGP.

Our study demonstrates that activation of TRPV1 increases the frequency of mEPSCs in control mice but not in type 1 diabetic mice, thereby indicating that at least one mechanism involved in the central regulation of liver-related preautonomic PVN neurons is dysfunctional in type 1 diabetic mice. One of the possible explanations for diminished TRPV1 response is a reduction in available membrane-bound TRPV1 receptors. Here, we did not find a significant change in total TRPV1 protein levels. However, we did not distinguish between membrane-bound and cytosolic TRPV1; thus, we cannot exclude a possible reduction of membrane-bound TRPV1 receptors. Observations from dorsal root ganglia cells of type 1 diabetic rats revealed decreased total TRPV1 protein levels, but the phosphorylation of TRPV1 and the amount of membrane-bound TRPV1 increased (44). This difference between the total TRPV1 expression levels could originate from the functional difference of sensory and central neurons, species difference, and that the animals were kept for a shorter time in our experiments. We also found an increased phospho-TRPV1–to–TRPV1 ratio in type 1 diabetic mice. Phosphorylation has three major effects: *1*) sensitization of TRPV1 (45); *2*) phosphorylation-dependent desensitization of TRPV1 (46); and *3*) induction of TRPV1 trafficking by protein kinase A (47). Our data indicate higher phosphorylation of TRPV1 in type 1 diabetic mice but without change in mEPSC frequency after capsaicin application, suggesting phosphorylation-dependent desensitization of TRPV1 (46). This is possible in the continuous presence of endogenous ligands (e.g., anandamide), suggested by increased levels of anandamide shown in obese and diabetic patients and animals (48,49), leading to desensitization to subsequent agonist challenges.

Our data revealed that either preincubation of the slices with insulin or in vivo insulin therapy of type 1 diabetic mice restored the TRPV1-dependent increase of mEPSC frequency in liver-related PVN neurons, suggesting a reconstitution of normal synaptic activity by insulin. Insulin, neuronal insulin receptors, and the insulin receptor substrate 2 are required for normal control of glucose homeostasis. Insulin receptor substrate 2 expression in the PVN and its colocalization with liver-related PVN neurons and TRPV1 (24) further support functional interaction between insulin and TRPV1 signaling. Insulin potentiates TRPV1 through a PI3K/PKC-dependent pathway, increases TRPV1 sensitivity via phosphorylation, increases density on the plasma membrane, and stimulates trafficking (31). Our data demonstrate that activation of PKC with PMA reinstated the TRPV1-dependent increase of mEPSC frequency in type 1 diabetic mice. This is consistent with our other observation that application of BIM, a PKC inhibitor, prevented the insulin-induced rescue effect on capsaicin-caused enhancement of mEPSC frequency, further supporting the involvement of PKC in TRPV1 activation and enhancement of glutamate release. The PI3K inhibitor, wortmannin, also prevented the insulin-dependent reinstatement of TRPV1; consequently, our findings support the hypothesis that insulin potentiates TRPV1 via a PI3K/PKC-dependent manner. Furthermore, insulin potentiates the recruitment of new TRPV1 into the plasma membrane by PKC (31). Our data support this observation because application of brefeldin A, a receptor trafficking inhibitor, prevented the insulin-caused reinstatement of TRPV1 activation. Based on these observations, we suggest that the diminished TRPV1 response of liver-related PVN neurons in type 1 diabetic mice is mediated by phosphorylation-dependent desensitization of TRPV1. Furthermore, insulin treatment restored the TRPV1 effect by induction of TRPV1 to the plasma membrane, which may result in an increase in density of new TRPV1 on the plasma membrane and sensitization of the new TRPV1 by insulin. In addition, loss of insulin signaling may have contributed to an internalization of TRPV1 receptors in synaptic terminals. Although numerous factors might be responsible for diminished TRPV1 function, chronically elevated glucose levels and/or diminished insulin levels seem likely to be the most critical features of the effect.

In conclusion, our data strongly support the hypothesis that disruption of the normal balance of the central autonomic circuitry in the PVN regulating the sympathetic and parasympathetic output to the liver is a pivotal factor in the development of autonomic imbalance ultimately resulting in enhanced HGP.

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H.G., K.M., and M.D.B. researched data. A.V.D. researched data and edited the manuscript. A.Z. designed the experiments, researched data, and wrote the manuscript. A.Z. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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