Prior hypoglycemia enhances glucose responsiveness in some ventromedial hypothalamic glucosensing neurons

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RECURRENT BOUTS of insulin-induced hypoglycemia (IIH) are common in patients with type 1 diabetes mellitus, especially children (3, 4, 23, 43). Such recurrent bouts lead to severe dampening of the hormonal counterregulatory and adrenomedullary responses (AMR) to subsequent bouts of hypoglycemia, a component of the clinical syndrome known as hypoglycemia-associated autonomic failure (2, 13, 53). In nondiabetic adult rats, we showed that a single bout of hypoglycemia results in impairment of AMR, in association with upregulation of glucokinase (GK) mRNA expression in the ventromedial hypothalamus (VMH) (15, 53). This hypothalamic area, which includes the arcuate (ARC) and ventromedial nuclei (VMN), contains glucosensing neurons, which we postulate to be critical elements in the detection of and response to hypoglycemia (8, 22, 25, 38, 48, 60). In contrast to most neurons in the brain, which utilize glucose to fuel their metabolic needs (47), glucosensing neurons utilize glucose as a signaling molecule to alter their membrane potential and firing rate (15, 22, 48, 60). Glucose-excited (GE) neurons increase, whereas glucose-inhibited (GI) neurons decrease, their firing rate as ambient glucose levels rise (15, 22, 28, 38, 48). Conversely, during low glucose availability, GI neurons are activated and GE neurons are inactivated. There is good evidence that neurons within the VMH play a critical role in the initiation of the AMR to hypoglycemia and the development of a dampened AMR, which occurs after IIH (6, 8, 9, 15, 33, 34, 53). A previous study in 2- to 3 wk-old rat pups showed that the development of a dampened AMR after three bouts of IIH was associated with a reduced responsiveness of VMN GI neurons to glucose (49). However, little is known about the actual mechanisms underlying such alterations in neuronal glucosensing and whether they occur in VMN GE neurons or other neurons outside the VMN. Our work (15, 21, 22), as well as that of others (60), strongly supports a role for GK as a key regulator of glucosensing in some neurons, similar to glucosensing in pancreatic β-cells (19, 31). Because a blunted AMR and increased VMH GK mRNA expression occur simultaneously 48 h, but not 24 h, after a single bout of IIH in adult, nondiabetic rats (15, 53), we postulated that increased GK activity in hypothalamic glucosensing neurons might make them more responsive to low glucose levels and underlie the reduced AMR in response to subsequent bouts of IIH.

Since type 1 diabetes mellitus is common in children and tight regulation of blood glucose levels in these children is associated with a reduced AMR to IIH (3, 4, 20), we developed a model of an attenuated AMR following recurrent IIH in juvenile rats to investigate potential mechanisms underlying this phenomenon. We used 4- to 5 wk-old rats to assess the timing of the blunted AMR, as well as changes in VMH expression of GK mRNA and the responsiveness of VMN and ARC glucosensing neurons to glucose, which might accom-
pany the blunted physiological responses. In addition, since previous studies demonstrated the importance of AMP-activated protein kinase (AMPK) in the AMR to hypoglycemia (33, 34) and glucosensing in GI (10) and GE (12) neurons, we also examined the mRNA expression of α1- and α2-subunits of AMPK in the VMH after a single bout of hypoglycemia. We predicted that prior hypoglycemia in juvenile rats would blunt the AMR, in association with increased GK mRNA and enzyme activity, which would lead to increased glucose responsiveness of VMH neurons.

MATERIALS AND METHODS

Animals and husbandry. All work was carried out under a protocol approved by the Institutional Animal Care and Use Committees of the New Jersey Medical School and the East Orange Veterans Affairs Medical Center and in accordance with the “Guiding Principles for Research Involving Animals and Human Beings” of the American Physiological Society (1). Male outbred 4- to 5-wk-old Sprague-Dawley rats (140–150 g body wt; Charles River Laboratories) were housed on a 12:12-h light-dark schedule (lights on at 0700) and kept at 22–23°C. Food (Purina rat chow no. 5001) and water were available ad libitum, except as noted below.

Assessment of the effect of a single episode of IHH on AMR to subsequent hypoglycemia. At 4 wk of age, rats (n = 8) were surgically implanted with jugular venous catheters and were maintained and handled daily postoperatively until they regained preoperative weight (~1 wk), as described previously (54). During this time, they were habituated to the experimental conditions in the procedure room. On the day of testing, food was withdrawn at 0800 and an initial blood sample (0.2 ml) was collected for measurement of baseline (~15 min) levels of plasma glucose and epinephrine. An equal volume of donor blood collected from unstressed rats was infused after each blood collection to maintain hemodynamics. Regular human insulin (Eli Lilly, Indianapolis, IN; 5 U/kg sc in 0.5 ml of saline) was administered, and blood samples (0.3 ml) for glucose and catecholamines were drawn 30, 60, 90, and 120 min later. After the 120-min blood sample was drawn, food was returned to all rats. At 24 h after the initial hypoglycemic episode, all rats were subjected to a second bout of hypoglycemia following the same protocol used for the first bout.

Plasma for the catecholamine assays was vortexed with 5 N perchloric acid and then immediately frozen on dry ice and held at −70°C for further processing. After all rats completed the protocol, the samples were briefly thawed and centrifuged, and 100 µl of supernatant were mixed with the internal standard dihydroxybenzylamine. Catecholamines were assayed using HPLC with electrochemical detection, as previously described (53). Plasma glucose was measured using a glucose analyzer (Analog Instruments, Lunenburg, MA).

Assessment of changes in VMH GK mRNA expression in response to a single bout of hypoglycemia. Additional groups of rats were injected subcutaneously with insulin (n = 6) as described above or with 0.5 ml of saline (n = 6). The insulin injections decreased tail blood glucose to 30–35 mg/dl over 120 min. After 24 h, the animals were killed, their brains were removed, and coronal hypothalamic sections (350 µm) through the midpoint of the ARC and VMN from rats injected with insulin (n = 6) or saline (n = 6) were cut on a Vibratome and then immediately transferred to RNAlater solution (Ambion, Austin, TX) for stabilization of RNA within tissues. The VMH (ARC + VMN) was dissected by hand using a scalpel under a surgical microscope to cut a triangular wedge, with the base of the triangle at the ventral surface of the hypothalamus. The lateral border was the sulus marking the lateral border of the ventral hypothalamic surface, and the apex of the triangle was the midpoint of the third ventricle. Using cresyl violet-stained sections in preliminary studies, we confirmed that these dissection planes include both the ARC and VMN. RNA was extracted using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA) and reverse transcribed with Superscript III with oligo(dt) priming and treated with RNase H (Invitrogen, Carlsbad, CA) (21, 27). The resultant cDNA was analyzed by real-time quantitative PCR on a Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) using primer/probe and FAM-labeled probe sets targeting pancreatic GK (GenBank accession no. NM012565), cyclophilin (GenBank accession no. M19533), hexokinase I (GenBank accession no. NM012734), and the catalytic subunits AMPKα1 (GenBank accession no. NM019142) and AMPKα2 (GenBank accession no. NM023991), as described previously (18, 21) (Table 1). Samples were compared against serially diluted reference standards prepared from pooled aliquots of cDNA from each sample. Data are expressed as the ratio of the standardized amount of the gene of interest to the standardized amount of cyclophilin, which is constitutively expressed and unaltered by hypoglycemia (data not shown).

Assessment of changes in glucose concentration-dependent responses of VMH neurons after a single bout of hypoglycemia. Rats were injected subcutaneously with insulin (n = 30) or saline (n = 30) as described above. Glucose levels measured 120 min after insulin or saline injection in tail blood fell to 30–35 mg/dl in all insulin-injected animals. After 24 h, rats were decapitated and their brains were rapidly removed. After the brain tissue was cut on a Vibratome, two to three 350-µm sections through the VMH (~2.5 to ~3.1 relative to bregma) were placed in a silicone-coated petri dish. Under a dissecting microscope, VMN (n = 15 per treatment group) or ARC (n = 15 per treatment group) nuclei were punched with a 500-µm blunt needle: the VMN was punched bilaterally; for the ARC, a single punch centered on the midline of the lower third of the third ventricle overlying the median eminence was made to include both nuclei. The resultant tissue punches were digested in papain (2 mg/ml, 30 min, 37°C) and mechanically triturated. Dissociated cells were plated onto coverslips and allowed to adhere for 60 min before intracellular Ca2+ concentration ([Ca2+]i) imaging, as described previously (15, 21, 22). Cells were loaded with the Ca2+ fluorophore fura 2-AM for 20 min in 2.5 mmol/l glucose-containing Hanks’ balanced salt solution buffer (in mmol/l: 135 NaCl, 5 KCl, 1 CaCl2, 1 MgCl2, and 10 HEPES, pH 7.4) at 37°C. Fura 2 fluorescence images were acquired every 5 s by alteration of excitation at 340 and 380 nm, and emissions (420–600 nm) were collected using a cooled, charge-coupled device camera (15, 22).

Dissociated VMN and ARC neurons were first classified by their [Ca2+]i changes in response to a decrease in extracellular glucose (from 2.5 to 0.1 mmol/l); those with a >20% decrease in area under the curve (AUC) of [Ca2+]i oscillations were classified as GE, those

Table 1. Primers for quantitative mRNA determinations

<table>
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<th>Forward</th>
<th>Reverse</th>
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<td>AMPKα2</td>
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</tr>
<tr>
<td>Glucokinase</td>
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<td>TCCGAGCTTTCTGAGCTTCTG</td>
<td>TCTACGCGGAAATTG</td>
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<td>Hexokinase I</td>
<td>CAGCTGCTGCTCAGATGCTT</td>
<td>CGAGATCCGACCCGCAATTGATC</td>
<td>TCTACGCGGAAATTG</td>
</tr>
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AMPK, AMP-activated protein kinase.
with a >59% increase in area under the curve (AUC) of [Ca^{2+}], oscillations as GI, and those that did not meet either of these specific criteria, which had been determined in our previous studies (22), as nonglucosensing. Although our previous studies were carried out at 0.3–2.5 mmol/l glucose, we used the lower glucose level (0.1 mmol/l) to cover the increase in glucose responsiveness that we predicted would occur due to the increase in hypoglycemia-associated elevations of GK expression. Then individual sets of neurons were exposed to various combinations of seven incremental changes (0.3, 0.5, 0.7, 1.0, 1.5, 2.0, and 2.5 mmol/l) of extracellular glucose concentrations. Because of the need to limit the duration of testing to avoid cell damage by the imaging laser, any given set of neurons was evaluated at only two to three of these increasing glucose concentrations. A minimum of 10 rats were used to assess neuronal responses for any set of 3 incremental glucose concentrations. Changes in [Ca^{2+}], oscillations from one condition to another were quantified as the AUC (22) and normalized by determination of the percentage of responses to a given concentration of glucose relative to the initial response at 2.5 mmol/l.

Statistics. Nadir plasma glucose and peak epinephrine levels, EC50, IC50, and maximum activation and inhibition values for glucose concentration-response curves, VMH GK, AMPKα1, and AMPKα2 (expressed as a function of the constitutive gene cyclophilin) were compared using an unpaired t-test. Plasma glucose and epinephrine levels during IIIH were also compared by one-way repeated-measures ANOVA with post hoc Bonferroni’s test. For neuronal glucosensing, AUC of [Ca^{2+}], oscillations was calculated using Origin software (OriginLab), and GE and GI neurons were defined using criteria established in our prior studies (22). EC50 and maximal activation values for GE neurons and IC50 and maximal inhibition values for GI neurons were determined by nonlinear regression analysis (sigmoidal dose-response curve fit; GraphPad Prism). For statistical comparison of the maximal activation and inhibition values, data from each neuron were calculated as the percent change from the AUC at 0.1 mmol/l to normalize for initial differences in AUC values at 0.1 mmol/l among neurons.

RESULTS

A single bout of IIIH impairs the AMR to subsequent hypoglycemia and alters VMH GK and AMPK mRNA expression. Insulin injections on day 1 caused blood glucose levels to decrease to a nadir of 34.5 ± 1.2 mg/dl and epinephrine levels to peak at 1,802 ± 294 pg/ml (Fig. 1). After a second injection of insulin on day 2, glucose fell to a significantly lower nadir (26.3 ± 0.1 mg/dl, P = 0.05) and peak epinephrine levels were significantly blunted (740 ± 81 pg/ml, P = 0.05) compared with levels on day 1 (Fig. 1). Also, repeated-measures ANOVA demonstrated lower glucose and epinephrine 30–120 min after the insulin injections on day 2, suggesting a significantly blunted AMR after repeated IIIH.

In association with this blunted AMR, VMH GK mRNA was increased by 46% in insulin- compared with saline-injected rats 24 h after the first injections (Table 2). This response was not generalized or nonspecific, since mRNA expression of hexokinase I and AMPKα1 was unchanged. Conversely, AMPKα2 expression was reduced by 18% in these same samples.

A single bout of hypoglycemia selectively increases glucose responsiveness of some VMH glucosensing neurons. We identified ARC [and VMN (trace not shown)] GE neurons as those that responded to a decrease from 2.5 to 0.1 mmol/l glucose with a decrease (Fig. 2A) and GI neurons as those that responded with an increase (Fig. 2B) of AUC of [Ca^{2+}], fluctuations. These responses were reversed in a concentration-dependent manner as glucose was raised in a stepwise fashion from 0.1 to 2.5 mmol/l (Figs. 3 and 4). Overall, a total of 800–1,442 neurons from 15 rats from each of the respective groups (VMN GE and GI and ARC GE and GI) were analyzed (Table 3). Using Ca^{2+} imaging, we previously demonstrated that VMN GE neurons from 4- to 5-wk-old rats (n = 8) 24 h after an initial bout on day 1. *P < 0.05.
Table 2. VMH hexokinase, glucokinase, AMPKα1, and AMPKα2 mRNA expression 24 h after a single injection of saline or insulin

<table>
<thead>
<tr>
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<th>Saline</th>
<th>Insulin</th>
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<tr>
<td>Hexokinase I</td>
<td>1.86±0.09</td>
<td>1.73±0.05</td>
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<tr>
<td>Glucokinase</td>
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</tr>
<tr>
<td>AMPKα1</td>
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<tr>
<td>AMPKα2</td>
<td>1.19±0.03</td>
<td>0.97±0.03*</td>
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Values are means ± SE, expressed as a function of cyclophilin mRNA values from the same micropunch samples. Insulin (5 U/kg) was injected to produce hypoglycemia. VMH, ventromedial hypothalamus. *P = 0.05 vs. saline (unpaired t-test).

mmol/l, P = 0.05; Fig. 3A, Table 3). Also, the maximum activation above the initial 0.1 mmol/l glucose was 31% higher in VMN GE neurons from insulin- than saline-injected rats. On the other hand, the maximal activation occurred at a higher concentration (1.5 mmol/l) in neurons from insulin- than saline-injected rats (0.7 mmol/l glucose; Fig. 3A, Table 3).

Although there was a tendency for a leftward shift in the responsiveness of ARC GE neurons from insulin-injected rats (EC50 = 0.21 mmol/l), this difference did not reach statistical significance compared with responses of neurons from saline-injected rats (EC50 = 0.35 mmol/l; Fig. 3B, Table 3). However, there was a significant 19% increase in the level of maximal activation, which occurred at 1.5 and 2.5 mmol/l glucose in ARC GE neurons from insulin- and saline-injected rats, respectively (Fig. 3B, Table 3). This suggests that prior hypoglycemia did increase the glucose sensitivity of ARC GE neurons. Also, we defined GE neurons as those with >20% decrease in AUC of [Ca2+]i fluctuations when glucose was decreased from 2.5 to 0.1 mmol/l (22). Thus increased glucose sensitivity could result in inhibition of fewer GE neurons from insulin-injected rats over this initial concentration range to explain why fewer ARC GE neurons were identified from insulin- than saline-injected rats (Table 3). However, if this were the case, it cannot explain why we did not find fewer VMN GE neurons.

By contrast to VMN GE neurons, VMN GI neurons from insulin-injected rats did not differ significantly from those from saline-injected rats in their glucose responsiveness as a function of their calculated IC50 values or maximal inhibition by glucose. Maximal inhibition occurred at a slightly higher glucose level in neurons from insulin-injected (2.5 mmol/l) than saline-injected (2.0 mmol/l) rats (Fig. 4A, Table 3). The calculated IC50 values for insulin-injected (0.12 mmol/l) and saline-injected VMN GI neurons (0.11 mmol/l) at 0.1–2.5 mmol/l glucose in the present study were an order of magnitude lower than those from untreated 4- to 5-wk-old rats tested previously using physiological glucose levels of 0.3–5 mmol/l (IC50 = 1.12 mmol/l) (21). Also, although EC50 values for VMN and ARC GE neurons from saline-injected rats were comparable, the calculated IC50 for ARC GI neurons (0.30 mmol/l) was threefold higher than that for VMN GI neurons (0.11 mmol/l) from saline-injected rats (Fig. 4, Table 3).

The glucose concentration-response curves for ARC GI neurons from saline- vs. insulin-injected rats were more difficult to compare. Although neurons from saline-injected rats followed the expected reduction in AUC of [Ca2+]i, as glucose was raised from 0.1 to 2.5 mmol/l (IC50 = 0.30 mmol/l), ARC GI neurons from insulin-injected rats reached maximal inhibition at 0.5 mmol/l but, from 0.7 to 2.5 mmol/l, AUC of [Ca2+]i was not inhibited with any predictable concentration-dependent relationship (Fig. 4, Table 3). The IC50 estimated for these neurons at 0.1–0.5 mmol/l was 0.18 mmol/l. Although these data suggest that ARC GI neurons from insulin-injected rats were two- to fivefold more sensitive to inhibition induced by increasing glucose concentrations than those from saline-injected rats, the two sets of values could not be compared...
The present studies utilized a juvenile rat model that mimics the blunted AMR, which occurs as a consequence of antecedent bouts of IIH (33, 34). We found that the dampened AMR was associated with increased VMH GK and decreased AMPKα2 mRNA expression and increased responsiveness of some, but not all, VMH glucosensing neurons to glucose. Although no definitive causal link was established, these results suggest a possible sequence of events whereby antecedent IIH increases GK expression in VMH glucosensing neurons. This would increase their glucose responsiveness, leading to a blunted AMR, because they would not respond fully at plasma glucose levels that activate this response in animals undergoing their first bout of hypoglycemia.

The most robust evidence for increased glucose responsiveness was seen in VMN GE neurons from insulin-injected rats. In these neurons, EC50 was reduced fourfold and maximal glucose-induced activation increased 31%, even though this maximum activation occurred at a higher glucose concentration than in saline-injected rats. In addition, although the EC50 was not significantly altered in ARC GE neurons following hypoglycemia, maximal activation occurred at lower glucose levels and was 19% higher than in saline-injected rats. There was a 10.220.33.1 on March 31, 2017 http://ajpregu.physiology.org/ Downloaded from

**DISCUSSION**

The full expression of AMR to hypoglycemia (6, 8, 9, 55) and, possibly, for the blunted AMR, which occurs after repeated bouts of IIH (33, 34). We found that the dampened AMR was associated with increased VMH GK and decreased AMPKα2 mRNA expression and increased responsiveness of some, but not all, VMH glucosensing neurons to glucose. Although no definitive causal link was established, these results suggest a possible sequence of events whereby antecedent IIH increases GK expression in VMH glucosensing neurons. This would increase their glucose responsiveness, leading to a blunted AMR, because they would not respond fully at plasma glucose levels that activate this response in animals undergoing their first bout of hypoglycemia.

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**Fig. 3. Glucose concentration-response curves for GE neurons from saline- and insulin-injected 4- to 5-wk-old rats. Rats were injected with saline or insulin (5 U/kg sc) and then killed 24 h later. Dissociated ventromedial nucleus (VMN, A) and ARC (B) neurons were held in 2.5 mmol/l glucose, and glucose levels were decreased to 0.1 mmol/l. Then glucose concentrations were increased in sets of 3 concentration increments between 0.3 and 2.5 mmol/l. Responsiveness was assessed as AUC of [Ca2+]i for individual neurons from insulin- vs. saline-injected rats. Neurons are defined as GE by decreased AUC of [Ca2+]i when glucose was decreased from 2.5 to 0.1 mmol/l and then by increased AUC when glucose was increased from 0.1 to 2.5 mmol/l.**

**Fig. 4. Glucose concentration-response curves for VMN (A) and ARC (B) GI neurons from insulin- vs. saline-injected rats. Conditions and data points are expressed as described in Fig. 3 legend, except IC50 for VMN GI neurons was calculated for inhibition of AUC of [Ca2+]i as glucose concentrations were increased from 0.1 to 2.5 mmol/l and IC50 for ARC GI neurons was calculated only from 0.1 to 0.5 mmol/l glucose.**

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neurons were then exposed to increasing concentrations of glucose. Neurons classified as GE or GI by their changes in intracellular Ca$^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) when glucose was lowered from 2.5 to 0.1 mmol/l glucose. Neurons were then exposed to increasing concentrations of glucose (0.1–2.5 mmol/l) for calculation of EC$\text{S}_{50}$ and maximal (Max) activation (GE neurons) and IC$\text{S}_{50}$ and maximal inhibition (GI neurons), % of Total, percentage of GE or GI neurons in each area per treatment group. *$P \leq 0.05$ vs. saline. †Estimate based on incomplete data at 0.1–0.5 mmol/l glucose.

<table>
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<th></th>
<th>% of Total</th>
<th>EC$\text{S}<em>{50}$/IC$\text{S}</em>{50}$ mmol/l</th>
<th>% of 0.1 mmol/l (baseline)</th>
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<tbody>
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A total of 3,786 neurons from saline- or insulin-treated rats (VMN from 642 saline- and 800 insulin-treated rats and ARC from 1,442 saline- and 902 insulin-treated rats) were classified as glucose excited (GE) or glucose inhibited (GI) by their changes in area under the curve of intracellular Ca$^{2+}$ concentration ($[\text{Ca}^{2+}]_i$) when glucose was lowered from 2.5 to 0.1 mmol/l glucose. Neurons were then exposed to increasing concentrations of glucose (0.1–2.5 mmol/l) for calculation (means ± SE) of EC$\text{S}_{50}$ and maximal (Max) activation (GE neurons) and IC$\text{S}_{50}$ and maximal inhibition (GI neurons). % of Total, percentage of GE or GI neurons in each area per treatment group. *$P \leq 0.05$ vs. saline. †Estimate based on incomplete data at 0.1–0.5 mmol/l glucose. was also a suggestion that ARC GI neurons might have become more sensitive to the inhibitory effects of glucose after hypoglycemia, but these data were more difficult to interpret. The fact that maximal inhibition occurred at a fivefold lower glucose concentration in ARC GI neurons from insulin- than saline-injected rats supports this contention. However, the maximal inhibition at 0.5 mmol/l was attenuated at higher glucose concentrations. This loss of inhibition is unexplained but was quite reproducible: it occurred with repeated trials in neurons subjected to a range of glucose concentrations (0.1–2.5 mmol/l). Unfortunately, this curious response prevented direct comparison with the results from saline-injected rats. Therefore, our overall results support the finding that VMN GE and, possibly, ARC GE neurons developed increased glucose responsiveness after a bout of hypoglycemia.

There are likely to be several different regulatory mechanisms utilized by neurons to sense glucose. Any of these might possibly, ARC GE neurons developed increased glucose responsiveness after a bout of hypoglycemia. As in the pancreatic β-cell (30), GK plays an important regulatory role in VMH neuronal glucosensing. For example, in the VMN, ~65% of GE and 45% of GI neurons express GK mRNA (22). Near-total inhibition of GK mRNA expression by RNA interference in primary VMH neuronal cultures almost completely abolishes glucosensing in GE and GI neurons, whereas pharmacological inhibition of GK activity inhibits GE and activates GI VMN neurons at 2.5 mmol/l glucose. On the other hand, pharmacological activation of GK enzyme activity has the opposite effect at 0.5 mmol/l (15, 21, 22).

Although such data imply that GK is a regulator of glucosensing in VMH neurons, it is likely that there are other mechanisms, such as AMPK, by which some neurons utilize glucose as a signaling molecule (10, 12), particularly during hypoglycemia (33, 34). AMPKα2 is particularly sensitive to alterations in brain glucose levels (37), and activation of AMPK is associated with an enhanced counterregulatory response to hypoglycemia (33, 34). Our finding of decreased expression of AMPKα2 in the VMH, in parallel with the development of a dampened AMR, suggests that these two events might be causally linked. Yet, because those VMH glucosensing neurons, which did alter their glucose sensitivity following IIH did so by increasing their responsiveness, it is less likely that decreased AMPKα2 activity would underlie this change. On the other hand, mRNA expression of AMPKα1 and AMPKα2 was increased and the counterregulatory response was dampened in adult rats after 3 days of recurrent bouts of IIH (33). However, these increases might represent a compensatory response several days after the initial downregulation of counterregulatory responses to hypoglycemia, which were seen here in juvenile rats 24 h and in adult rats 48 h after a single bout of hypoglycemia (53). Thus the increase in VMGK mRNA associated with a possible parallel increase in GK activity is a likely explanation for the increased responsiveness of VMH glucosensing neurons to glucose after IIH.

An unexpected finding of the present studies was the leftward shift in responsiveness of VMN and ARC GI neurons isolated from saline-injected control rats compared with our previous studies (21, 48, 59). One potential reason for this outcome is that the original studies were carried out at glucose levels between 0.3 or 0.5 mmol/l and 2.5 mmol/l. In fact, 0.1 mmol/l glucose, to which neurons were exposed in the present study, is far below levels seen during even the most severe hypoglycemia (14). Thus exposure of isolated GI neurons to such low glucose levels may have acutely altered their underlying glucosensing capacity and subsequent responses to raising glucose levels again. It is possible that GI neurons were selectively affected by such exposure, because they normally respond to glucose at levels almost twice as high (IC$\text{S}_{50} = 1.12$ mmol/l) as those at which GE neurons respond (IC$\text{S}_{50} = 0.54$ mmol/l), at least in the VMN (21). Although patch-clamped neurons in VMN slices from 2- to 3-wk-old rats showed no such effect of lowering glucose to 0.1 mmol/l (49), those neurons could utilize lactate from adjacent astrocytes to fuel their metabolic needs (52), whereas neurons in our studies were isolated from astrocytes. In addition, we utilized $[\text{Ca}^{2+}]_i$ fluctuations as our index of changes in glucose responsiveness. Although we previously demonstrated that such fluctuations correlate well with glucose-induced changes in membrane potential (21), it is still possible that this association breaks down after exposure of isolated neurons to very low levels of glucose. Altered neuronal function and/or a dissociation between $[\text{Ca}^{2+}]_i$, and membrane potential fluctuations to glucose might result from disordered mitochondrial electron transport chain function during hypoglycemia due to the decreased availability of reducing equivalents. This would increase mitochondrial production of free radicals and lead to mitochondrial oxidant injury (24).

The present studies also differ in other respects from the only other study in which VMH glucosensing neurons were examined after recurrent bouts of IIH (49). The previous study (49) demonstrated that VMN GI neurons from recurrently hypoglycemic rats failed to respond at 0.5 mmol/l glucose but did respond at 0.1 mmol/l, suggesting that recurrent hypoglycemia...
cemia had made them less, rather than more, responsive to glucose. However, there are several differences between these two studies. First, the previous study was carried out in 14- to 21-day-old pups subjected to three bouts, rather than one bout, of antecedent IIH, where glucose levels ranged from 1.4–2.3 mmol/l blood glucose. Also, as mentioned above, glucosensing neurons were assessed using the patch-clamp technique in VMN slices, for which astrocytic glycogen was presumably available as a temporary buffer against severe hypoglycemia (49, 52). In addition, 14–21 days is the age at which the primary source of cerebral energy supply changes from ketone bodies derived from maternal milk to glucose derived from ingested food. This transition is paralleled by a switch from a heavy reliance on the transport of ketone bodies into the brain and neurons by monocarboxylate transporters to transport of glucose by glucose transporters (40, 56, 57). Thus differences between the previous (49) and present results are not surprising given the differences in the age, degree of hypoglycemia, source of energy for neuronal metabolism, and mode of testing.

One important caveat of the present studies is that we measured only GK and AMPK mRNA levels and did not assess the activity of either enzyme. This is particularly important with regard to GK, for which we have been unable to find an experimentally reproducible activity assay. This is likely due to the extremely low abundance of GK mRNA expression in the VMH and individual VMH neurons (15, 22). These levels are so low that it has not been possible to reliably demonstrate the presence of GK protein in the VMH, as opposed to the hindbrain, where GK immunoreactive neurons and GK mRNA have been demonstrated (26, 29). Thus we have not been able to establish a conclusive link between increased GK mRNA expression and a parallel increase in GK activity in our studies.

In conclusion, our findings indicate that a single bout of IIH in juvenile rats leads to a blunted AMR in association with increased VMH GK mRNA expression and increased glucose responsiveness of a subpopulation of VMH glucosensing neurons. The hypothetical model derived from these studies is that hypoglycemia induces some VMH glucosensing neurons to produce more GK mRNA, leading to increased GK activity. This predicts that these neurons would not respond until glucose levels fell to lower levels and/or would not be as strongly activated at comparable glucose levels as control neurons subjected to their first bout of IIH. These leftward and upward shifts in responsiveness could contribute to the dampening of the AMR under such circumstances. However, it is also likely that many different types of VMH (and other brain area) glucosensing neurons utilize additional regulatory mechanisms for neuronal glucosensing, which might contribute to the blunting of AMR after hypoglycemia. Our present and previous data further demonstrate that hypoglycemia affects neurons in the ARC and VMN (53), each of which has its own spectrum of neurotransmitters, neuropeptides, and neuronal projections. The important implication of such results is that therapies aimed at preventing the development of hypoglycemia-associated autonomic failure or increasing the counterregulatory response to IIH in type 1 diabetic patients may have to take into account the heterogeneous characteristics of the neuronal populations in the critical hypothalamic area.

**Perspectives and Significance**

A comprehensive series of studies from investigators at Yale Medical School dating back to 1994 have established the VMH as a critical site for the control of the counterregulatory responses to IIH (6–9, 11, 16, 32–35). Although components of these responses can be elicited by glucoprivic stimuli applied to a variety of hindbrain sites (42), such responses do not occur without an intact VMH (44, 45). Although we have focused on the alterations in glucose responsiveness of isolated VMH glucosensing neurons caused by antecedent hypoglycemia, the picture is obviously much more complicated. First, the function of these neurons cannot be separated from their critical symbiotic relationship with astrocytes, which provide metabolic substrate and, in turn, are provided with energy sources such as glutamate from neurons (39, 52). Second, glucosensing neurons in the VMH are quite heterogeneous with regard to the mechanisms that they utilize to sense glucose, their transmitter and peptide phenotypes, and the myriad metabolic, neurohumoral, and presynaptic inputs that impinge on them (17, 22, 46, 48). Transmitters such as noradrenaline (5, 41), GABA (5, 11), and glutamate (55) and neuropeptides such as neuropeptide Y (59), corticotrophin-releasing factor, and urocortin (35) act as important modulators of neuronal activity in the VMH, the counterregulatory responses to hypoglycemia, and the dampening of these responses that follow even a single bout of hypoglycemia. In addition, glucosensing neurons respond directly to a variety of hormones such as insulin (22, 51, 59) and leptin (28, 50) and the availability of alternate fuels such as lactate (49) and fatty acids (36, 58). Thus, although we demonstrate here that antecedent hypoglycemia directly alters the glucose responsiveness of VMH glucosensing neurons, it also affects many of these other critical inputs to these neurons. Whereas many of these changes represent physiological adaptations to prior hypoglycemia, the end result of dampened counterregulatory responses can be life threatening. Our challenge is to construct an integrated model of how the brain controls the counterregulatory responses to hypoglycemia with the aim of developing medical therapies to prevent the serious consequences of recurrent hypoglycemia in the diabetic patient.

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