

# Metabolic Pathways That Mediate Inhibition of Hypothalamic Neurons by Glucose

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Neurons in the ventromedial hypothalamus mediate some counterregulatory responses to hypoglycemia and 2-deoxyglucose, but the mechanisms that mediate these responses to glucose are unclear. In the present study, ventromedial hypothalamus neurons were identified on the basis of their inhibition by the transition from 5 to 20 mmol/l glucose. Tolbutamide, which activates glucose-stimulated neurons, failed to inhibit or activate glucose-inhibited neurons. Inhibitors of glucose transport and glycolysis, in particular by the glucokinase inhibitor glucosamine, blocked the effect of glucose on glucose-inhibited neurons. Furthermore, the glucose-inhibited neurons were activated by 2-deoxyglucose, which also activates counterregulatory responses. Conversely, glucose-inhibited neurons were inhibited by glycolytic metabolites, including lactate, but not by pyruvate. These data indicate that hypoglycemia induces electrical activity in glucose-inhibited neurons by attenuating glycolysis in those neurons. Thus, counterregulatory failure could be due to relatively enhanced glycolysis in glucose-stimulated neurons during hypoglycemia and attenuation of glycolysis in glucose-inhibited neurons might reverse counterregulatory failure. *Diabetes* 53:67–73, 2004

**F**ailure in the counterregulatory responses to hypoglycemia is considered to be a limiting factor in the optimal treatment of type 1 diabetes (1). The ventromedial hypothalamus (VMH) seems to mediate some counterregulatory responses to hypoglycemia because local attenuation of glucose metabolism by 2-deoxyglucose (2-DG) in the VMH stimulates peripheral counterregulatory responses (2). Furthermore, counterregulatory responses to systemic glucopenia are blocked by lesions in the VMH (3) and by infusion of glucose into the VMH (4). Although some neurons in the VMH are activated by increasing glucose concentration, other VMH neurons are directly inhibited as glucose increases (5,6). Neurons that are sensitive to glucose at plasma concentrations (~3 to ~10–20 mmol/l glucose) are referred to as

glucose stimulated and glucose inhibited, respectively (7). Glucose-inhibited neurons are presumably activated by and may mediate some responses to hypoglycemia. Although glucokinase may mediate effects of glucose to inhibit VMH neurons (6), as stated by Schuit et al. (7), “relatively little is known about the mechanism underlying this type of glucose inhibition.” Extensive studies of pancreatic  $\beta$ -cells provided key guidance to studying mechanisms of glucose stimulation of VMH neurons (7,8). However, much less is known about how glucose inhibits, for example, glucagon-producing  $\alpha$ -cells, and inhibition of glucagon secretion by glucose seems to be complex and may involve indirect mechanisms (9,10). Of particular interest, in intact islets, metabolic pathways that regulate glucagon secretion seem to be distinct from those that regulate insulin secretion (11). Therefore, in the present studies, we used methods previously used to map metabolic pathways in glucose-stimulated neurons to assess pathways that mediate effects of glucose to inhibit hypothalamic neuronal activity.

## RESEARCH DESIGN AND METHODS

**Brain slice preparation.** Male Sprague-Dawley rats, 200–300 g body wt, were obtained from Charles River. Rats were housed using reversed light/dark cycle, with ad libitum access to food and water. For preparing brain slices, rats were anesthetized by intraperitoneal injection of urethane (1.6 g/kg) followed by decapitation. Brains were promptly removed and placed in ice-cold PBS buffer. Thin coronal slices (400  $\mu$ m) containing the VMH were cut with a Vibratome (Lancaster, Series 5000). Three slices were obtained per hypothalamus and each slice was cut bilaterally to obtain six slices per hypothalamus. Slices were placed into sucrose artificial cerebrospinal fluid in which sucrose replaced an equal molar concentration of NaCl of regular artificial cerebral spinal fluid (R-ACSF) (producing a temporary concentration of 0 mmol/l NaCl and a sucrose concentration of 248 mmol/l, see below) for a 1-h recovery period at room temperature. This was followed by transferring into R-ACSF (in mmol/l) 124 NaCl, 26 NaHPO<sub>4</sub>, 5 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.3 MgSO<sub>4</sub>, 2.4 CaCl<sub>2</sub>, and 10 D-glucose. In producing test solutions based on R-ACSF that involved altering the concentration of glucose or other components, osmolarity was maintained constant by adjusting NaCl concentration. All solutions were saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, maintained at pH 7.4 and at 34°C before perfusing to the recording chamber.

**Electrophysiologic recording.** Glucose-inhibited and nonresponsive neurons in VMH were studied by extracellular single-cell recording as previously described for studying glucose-stimulated neurons (8). Briefly, the slice was laid on a net in a 2-ml recording chamber in which warm (34°C) ACSF at the rate of 2 to 3 ml/min was circulated. Glass microelectrodes exhibiting a resistance of at least 5 to 10 M $\Omega$  were used to carry out single-cell extracellular recording. Action potentials were recorded by conventional electrophysiologic methods, amplified by an Axoclamp 2A amplifier (Axon Instruments), and the firing rate histograms were compiled and displayed. Data were recorded on videotape and computer for further analysis. Although the glucose concentrations to which glucose-sensitive hypothalamic neurons are actually exposed remains controversial (12), glucokinase is likely to serve as an important glucose-sensing mechanism when glucose concentrations are above ~5 mmol/l (13). Furthermore, we previously observed that neurons that respond to the transition from 5 to 20 mmol/l are observed in the hypothala-

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ACSF, artificial cerebral spinal fluid; 2-DG, 2-deoxyglucose; R-ACSF, regular ACSF; VMH, ventromedial hypothalamus.

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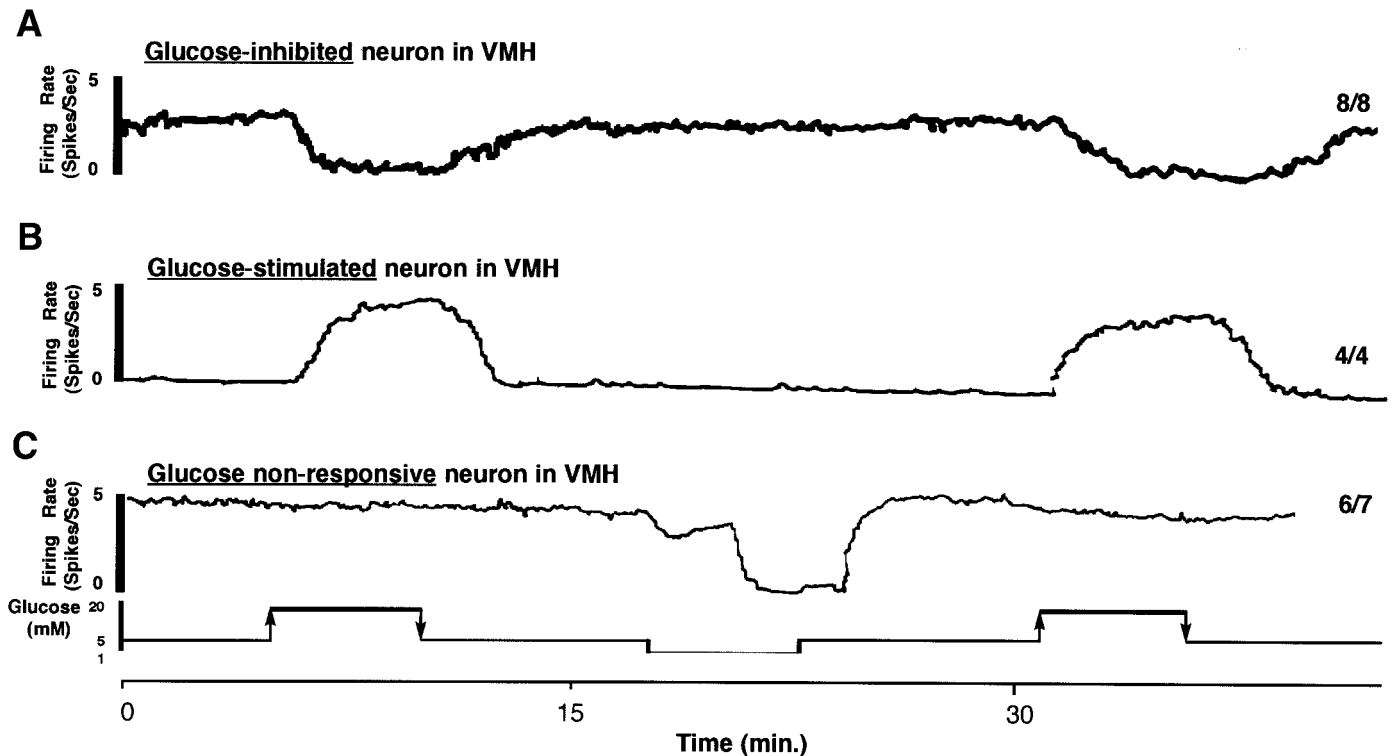


FIG. 1. Neurons in the VMH are inhibited by (glucose inhibited), stimulated by (glucose stimulated), or do not respond to (glucose nonresponsive) the transition from 5 to 20 mmol/l glucose. *A*: Glucose-inhibited neuron, active at 5 mmol/l glucose and silent at 20 mmol/l glucose. All glucose-inhibited neurons reported in this article were characterized by this behavior but constituted only ~34% of the total number of VMH neurons examined. *B*: As reported previously, some neurons in the VMH were activated by the transition from 5 to 20 mmol/l glucose and thus were referred to as glucose stimulated. *C*: Glucose-nonresponsive neurons did not respond to the transition from 5 to 20 mmol/l glucose. However, such neurons did become much less active when the glucose concentration decreased from 5 to 1 mmol/l glucose (numbers in upper right corner of each panel: denominator indicates the number of cells tested according to the protocol indicated for that panel, and numerator indicates the number of cells that behaved according to the salient behavior of that panel).

mus but not the cortex (8). In contrast, cortical and hippocampal neurons do not respond to changes in glucose concentration between 3.6 and 17 mmol/l glucose but do respond to changes in glucose concentrations <2 mmol/l (12,14). We therefore chose to focus in the present study on neurons that were sensitive to transitions from 5 to 20 mmol/l. For detecting glucose-inhibited neurons, slices were initially examined at 10 mmol/l glucose. An electrode was targeted to the VMH and slowly lowered until an active cell was detected by an extracellular recording that indicated repeating action potentials. The ACSF was then switched to 5 mmol/l glucose. When the activity remained stable for 5 min, the ACSF was then switched to 20 mmol/l. When the neuron became silent within 2 to 5 min, the neuron was provisionally classified as glucose inhibited. When the cell became electrically active once more after switching to 5 mmol/l glucose from 20 mmol/l glucose, further assessment of the neuron was carried out. Test agents were applied by switching the recording chamber perfusion to an ACSF containing the test solution in either 5 or 20 mmol/l glucose. The average perfusion time of the test reagent was ~5 min for each agent.

**Reagents.** 2-DG, glucosamine, mannose, galactose, glyceraldehyde, phloridzin, iodoacetic acid, glycerol, lactate, norepinephrine, and pyruvic acid (sodium salt) all were purchased from Sigma (St. Louis, MO).

## RESULTS

**VMH neurons inhibited by the transition from 5 to 20 mmol/l glucose.** In the present study, 34% of the 638 neurons examined were inhibited when glucose concentration increased from 5 to 20 mmol/l (Fig. 1A), 18% were stimulated by the transition from 5 to 20 mmol/l (Fig. 1B), and 48% of the neurons did not respond to the transition from 5 to 20 mmol/l (Fig. 1C). Hypothalamic neurons inhibited by the transition from 5 to 20 mmol/l glucose were first described in 1982 (15) and referred to as

glucose-sensitive neurons; however, to avoid confusion, these neurons are referred to herein by a more informative term: glucose inhibited (7).

**Glucose-inhibited neurons not sensitive tolbutamide.** We had previously demonstrated that every neuron activated by the transition from 5 to 20 mmol/l glucose is also activated by K-ATP channel blocker tolbutamide (8), and Song et al. (5) previously demonstrated that neurons inhibited indirectly by glucose (by activation of glucose-stimulated neurons) were also inhibited by tolbutamide. Therefore, it was of particular interest that neurons inhibited by the transition of 5 to 20 mmol/l glucose were not inhibited by tolbutamide (Fig. 2A). Nevertheless, as previously reported, tolbutamide activated neurons that are stimulated by the transition from 5 to 20 mmol/l (Fig. 2B). Furthermore, tolbutamide activated glucose-nonresponsive neurons that were inhibited by the K-ATP channel opener diazoxide (Fig. 2C) but otherwise did not influence the activity of neurons that were not sensitive to glucose (Fig. 2D). These observations are consistent with the hypothesis that glucose-inhibited neurons may not be inhibited by glucose-stimulated neurons.

**2-DG activates glucose-inhibited neurons.** Because local infusion of 2-DG into the VMH activates counterregulatory responses (2), it was of interest to assess whether glucose-inhibited neurons are activated by 2-DG. First, we observed that 2-DG blocked the effect of glucose to inhibit these neurons (Fig. 3A). Second, we observed that at 20

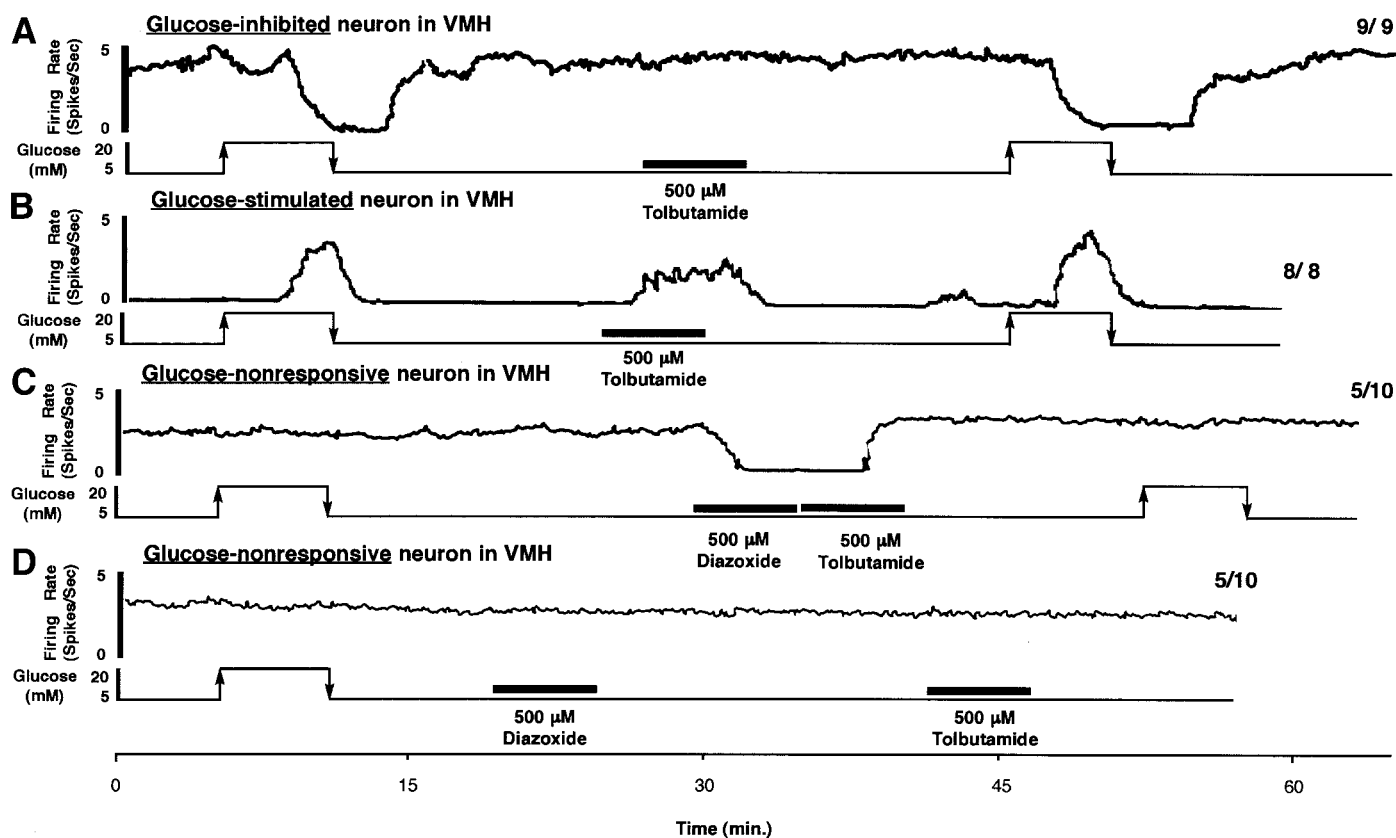


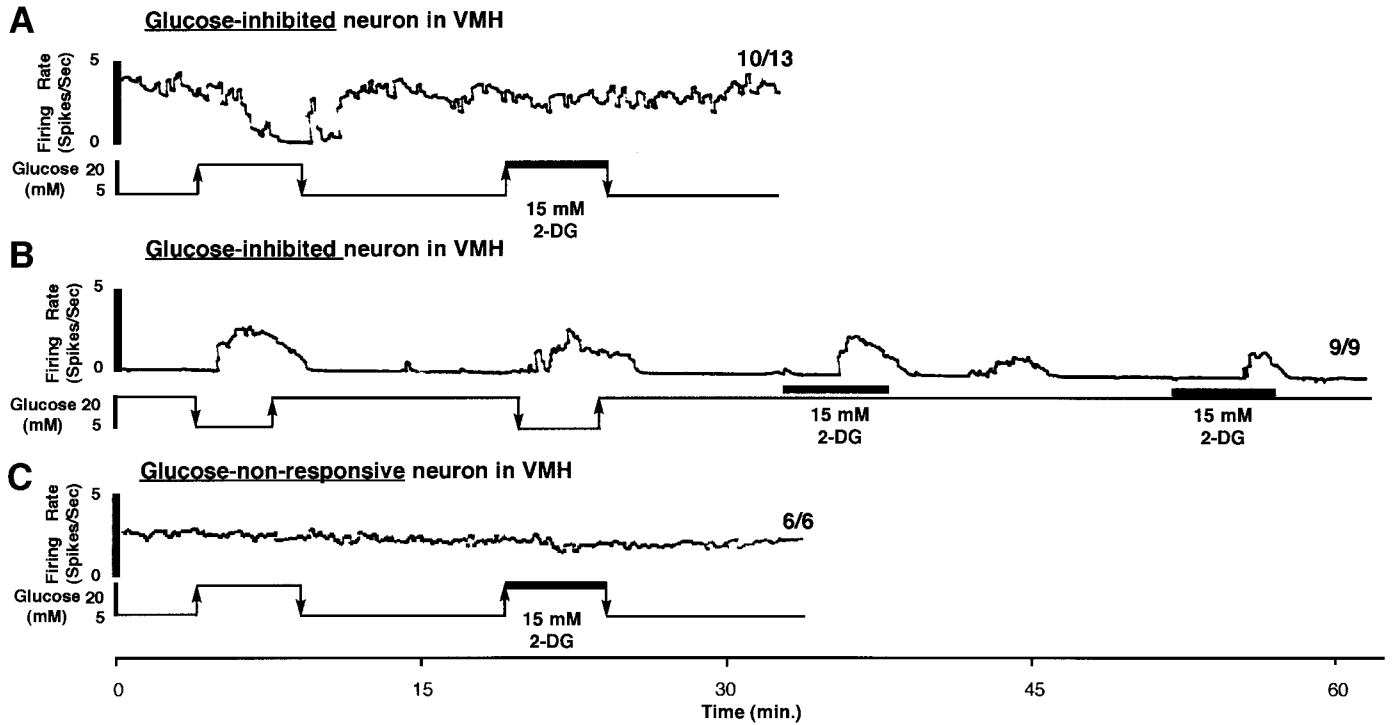
FIG. 2. Tolbutamide does not inhibit glucose-inhibited neurons but does stimulate glucose-stimulated neurons. *A*: Glucose-inhibited neurons are not inhibited (or stimulated) by tolbutamide. *B*: Glucose-stimulated neurons are stimulated by tolbutamide. Tolbutamide activated the three neurons in which diazoxide blocked the effect of glucose; diazoxide also blocked the stimulatory effects of norepinephrine, indicating that the inhibitory effect of diazoxide was nonspecific. *C*: Diazoxide inhibited the resting activity of 5/10 glucose-nonresponsive neurons, and this inhibition was reversed by tolbutamide. *D*: In neurons not sensitive to diazoxide, tolbutamide had no effect.

mmol/l glucose, 2-DG activated these neurons (Fig. 3*B*). In contrast, the same doses of 2-DG produced no effect in glucose-nonresponsive neurons (Fig. 3*C*).

**Inhibitors of glucose transport and metabolism block the inhibitory effect of glucose on glucose-inhibited neurons.** We have previously reported that the glucokinase inhibitor glucosamine blocks the effect of glucose on glucose-stimulated neurons (8), and Dunn-Meynell et al. (6) reported that glucokinase inhibitors activated isolated neurons inhibited by the transition from 0.5 to 2.5 mmol/l. We similarly observed that glucosamine blocked the effect of glucose on glucose-inhibited neurons (Fig. 4*A*). Glucosamine did not block the effect of norepinephrine on these neurons (Fig. 4*A*); we have previously reported that glucosamine at this dose does not influence electrical activity of glucose-nonresponsive neurons (8). We have previously reported that phloridzin, which blocks glucose transport through sodium-glucose cotransporters, blocks the stimulatory effect of glucose on glucose-stimulated hypothalamic neurons (8); phloridzin also blocks pancreatic responses to glucose (16). In the present study, 1 mmol/l phloridzin also blocked the effect of glucose on glucose-inhibited neurons (Fig. 4*B*). We have previously reported that the same dose of phloridzin has minimal effect on glucose-nonresponsive neurons (8). The previous results suggested that transport and phosphorylation of glucose are necessary for the activation of VMH neurons by the transition from 5 to 20 mmol/l glucose. For assess-

ing the requirement for later steps in glycolysis, the effect of iodoacetic acid, which, at appropriate doses, is reported to specifically inhibit glyceraldehyde phosphate dehydrogenase, was assessed. This step is notable in that it is the only step in glycolysis that produces NADH. In the present study, 0.2 mmol/l iodoacetic acid blocked the effect of glucose on glucose-inhibited neurons; this effect was not reversed 15 min after removal of the iodoacetic acid (Fig. 4*C*). In contrast, at the dose used, iodoacetic acid had no effect on the resting activity of glucose-nonresponsive neurons (8).

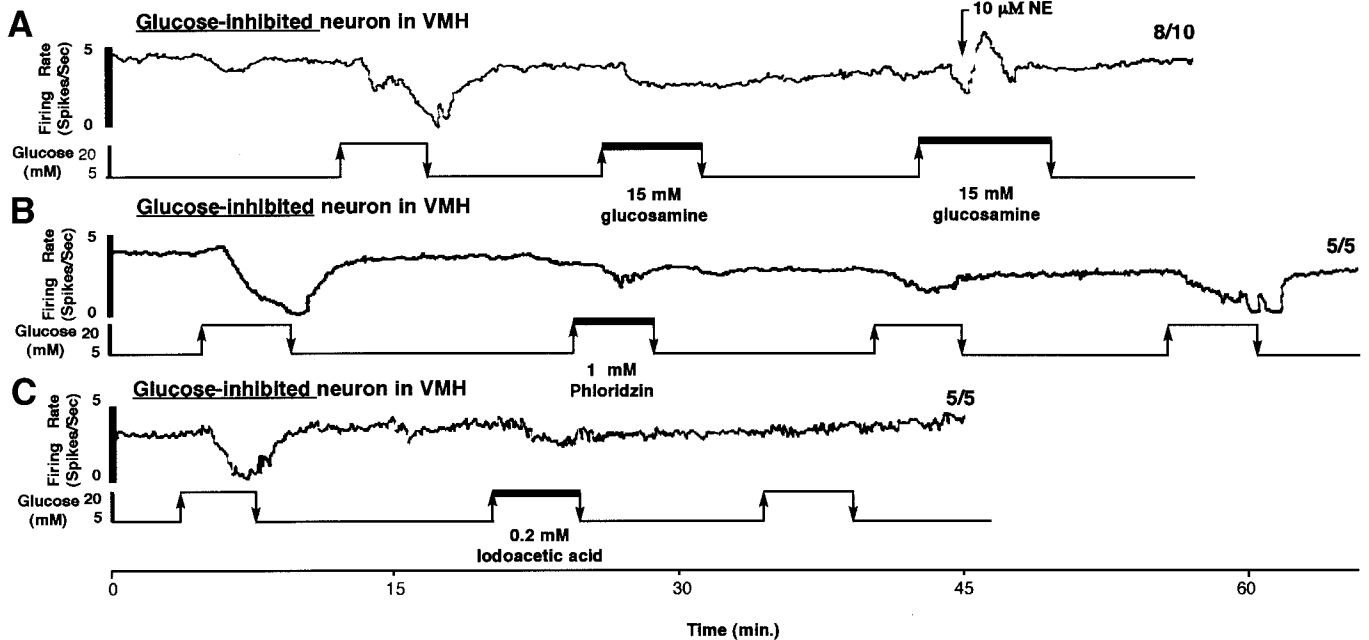
**Early glycolytic metabolites but not pyruvate mimic glucose effect on glucose-inhibited neurons.** The studies with inhibitors, described above, indicated that glycolytic steps up to and including the dehydrogenation of glyceraldehyde-3-phosphate are necessary for the effect of glucose on glucose-inhibited neurons. For assessing which metabolites are sufficient to mediate the effect of glucose on glucose-inhibited neurons, the following studies assessed the effects of 15 mmol/l of glycolytic metabolite (in the presence of 5 mmol/l glucose) on VMH firing rate. The addition of 15 mmol/l galactose (Fig. 5*A*), glyceraldehyde (Fig. 5*A*), mannose (Fig. 5*B*), or glycerol (Fig. 5*C*) to 5 mmol/l glucose mimicked the effect of 20 mmol/l glucose on glucose-inhibited neurons; these metabolites have no effect on glucose-nonresponsive neurons (8). These results were of interest because, although consistent with the role of



**FIG. 3.** 2-DG blocks the effect of glucose on glucose-inhibited neurons and stimulates glucose-inhibited neurons at 20 mmol/l glucose. *A*: In 10/13 glucose-inhibited neurons, simultaneous administration of 2-DG with glucose (filled bar) blocked inhibitory effects of glucose. *B*: At 20 mmol/l glucose, 2-DG (filled bar) activated all nine glucose-inhibited neurons tested. *C*: At the same dose, 2-DG had no effect on glucose-nonresponsive neurons.

glycolytic intermediates in sensing glucose, neither galactose nor glycerol stimulates pancreatic  $\beta$ -cells (16,17). Thus, in this respect, glucose-inhibited VMH neurons differ from pancreatic  $\beta$ -cells. However, we previously reported that these intermediates stimulate

glucose-stimulated neurons (8). Although early glycolytic intermediates could mimic the effect of glucose on glucose-inhibited neurons, we have observed that pyruvate fails to mimic the effect of glucose on glucose-stimulated VMH neurons (8). Similarly, pyruvate fails to



**FIG. 4.** Inhibitors of glucose transport and metabolism block inhibitory effects of glucose. The inhibition of glucose-inhibited neurons by glucose is blocked by glucosamine (*A*), an inhibitor of glucokinase that did not block stimulatory effects of norepinephrine (NE); phloridzin (*B*), an inhibitor of sodium-glucose cotransporters, the effect of which is reversed after ~30 min of washout; and iodoacetic acid (*C*), an inhibitor of glucose-6-phosphate dehydrogenase.

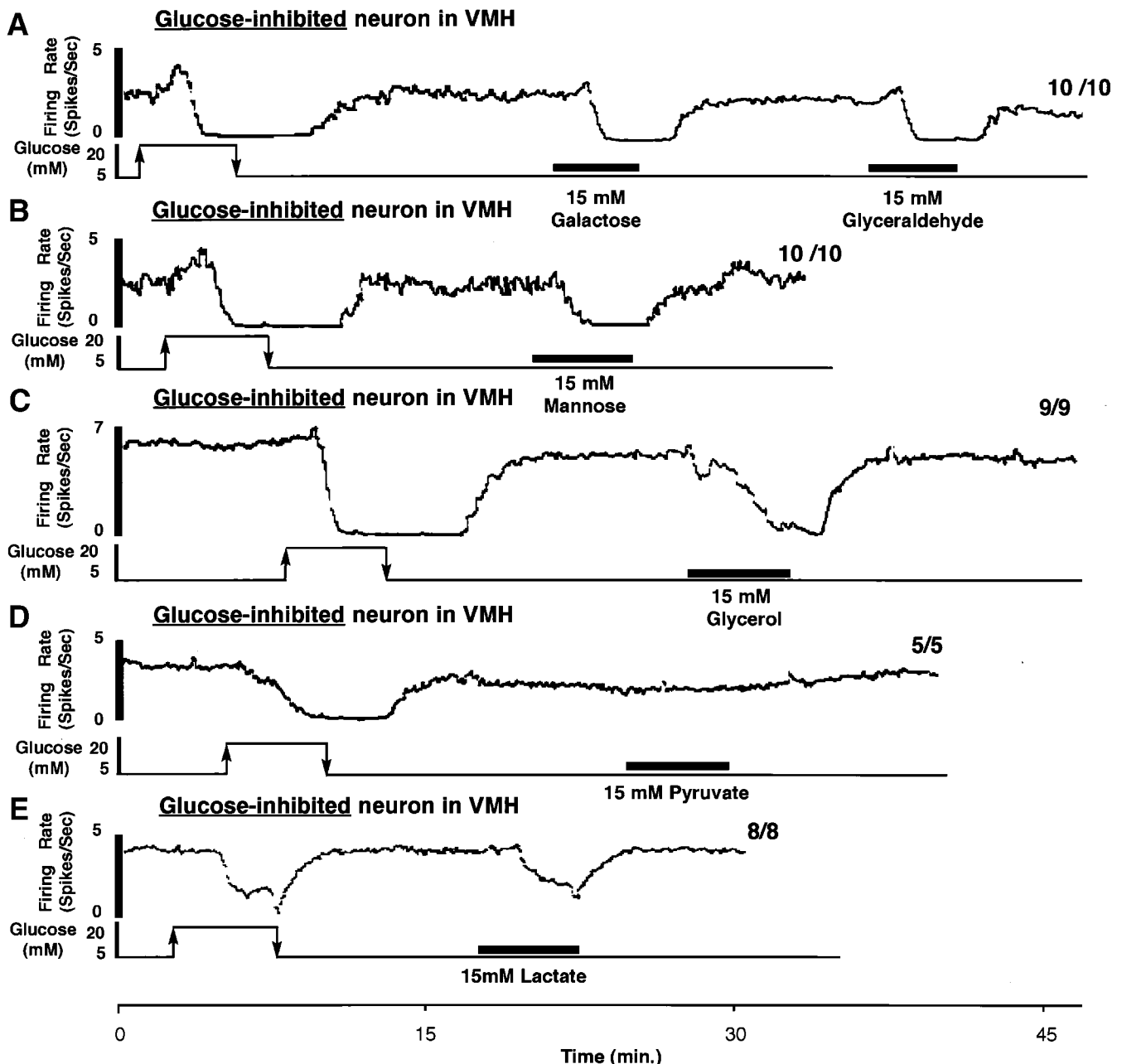


FIG. 5. Glycolytic metabolites, except pyruvate, mimic effects of glucose. Glucose-inhibited neurons are inhibited by galactose and glyceraldehyde (A), mannose (B), glycerol (C), and lactate (E). Glucose-inhibited neurons are not inhibited by pyruvate (D).

mimic the effect of glucose on pancreatic  $\beta$ -cells (16). Consistent with these observations, 15 mmol/l pyruvate plus 5 mmol/l glucose failed to inhibit glucose-inhibited neurons (Fig. 5D). It should be noted that we have previously demonstrated that VMH neurons can metabolize pyruvate (8).

The failure of pyruvate to stimulate glucose-stimulated neurons is consistent with observations in pancreatic  $\beta$ -cells (18,19). In pancreas, this observation has led to the conclusion that production of ATP from oxidative metabolism of pyruvate, the main source of ATP, may not be required for stimulation of insulin secretion by glucose. It has also been reported that lactate fails to stimulate pancreatic  $\beta$ -cells (19), which may be due to the inability

of pancreatic  $\beta$ -cells to metabolize lactate (20). Consistent with these results, lactate dehydrogenase activity seems to be low in pancreatic  $\beta$ -cells (21). In contrast, neurons can metabolize lactate preferentially, consistent with the expression in neurons of the heart form of lactate dehydrogenase, which preferentially converts lactate to pyruvate (22). Therefore, it was of interest to assess whether lactate could mimic the effect of glucose on glucose-inhibited neurons. In marked contrast to pyruvate and in contrast to pancreatic  $\beta$ -cells, addition of 15 mmol/l lactate plus 5 mmol/l glucose inhibited neurons that were also inhibited by the transition from 5 to 20 mmol/l glucose (Fig. 5E). In contrast, addition of 15 mmol/l lactate had no effect on glucose-nonresponsive neurons (8).

## DISCUSSION

Infusion of 2-DG into the VMH activates systemic counterregulatory responses (2), and destruction of VMH neurons blocks responses to 2-DG (3). These observations strongly suggest that neuroendocrine responses to hypoglycemia are mediated by neurons in the VMH that are activated by hypoglycemia and thus inhibited by elevated glucose. In contrast to cortical and hippocampal neurons, hypothalamic neurons respond to glucose concentrations in the range of plasma glucose (5–20 mmol/l) (12) and glucokinase is maximally effective as a glucose sensor in this range (13). Therefore, in the present study, we focused on neurons inhibited when glucose concentration increases from 5 to 20 mmol/l (or, equivalently, activated when glucose concentration decreases from 20 to 5 mmol/l); these neurons are referred to as glucose inhibited (7).

The present study indicates that glucose-inhibited neurons sense glucose through changes in glucose metabolism, similar to mechanisms that mediate effects of glucose on pancreatic and other glucose-sensing cells (7,8,13,19). Thus, inhibitors of glucose transport and glycolysis block effects of glucose (Figs. 3 and 4), and glycolytic intermediates mimic effects of glucose (Fig. 5) on glucose-inhibited neurons. Therefore, the metabolic pathways that mediate the inhibitory effects of glucose seem to be similar to those that mediate the stimulatory effect of glucose on VMH neurons (8) and pancreatic  $\beta$ -cells (16). In particular, the effect of glucosamine, an inhibitor of glucokinase, to block effects of glucose on glucose-inhibited neurons is consistent with the role of glucokinase as a key glucose-sensing molecule in hypothalamic neurons (6,8) and other neuroendocrine glucose-sensing cells (7,13). Furthermore, because lactate but not pyruvate mimicked the effect of glucose on glucose-inhibited neurons, cytoplasmic production of NADH (generated by the production of pyruvate from lactate) seems to be a more important signal mediating the inhibitory effect of glucose than mitochondrial production of ATP from pyruvate. This observation is consistent with our observations in glucose-stimulated hypothalamic neurons (8). Other recent studies corroborated these observations and also indicate that acute glucose does not detectably influence hypothalamic neuronal ATP levels but does increase neuronal NADH/NADPH (23). These results are also consistent with evidence that glucose-stimulated insulin secretion in pancreatic  $\beta$ -cells requires the cytoplasmic production and import of NADH into the mitochondria (24). It is of particular interest that lactate can substitute for glucose to block counterregulatory responses mediated by the VMH (25), supporting the hypothesis that VMH neurons inhibited by lactate mediate hypoglycemia-induced counterregulatory responses. Furthermore, the effect of glucose on glucose-inhibited neurons was blocked by 2-DG (Fig. 3A), every glucose-inhibited neuron was stimulated by 2-DG, and every 2-DG-stimulated neuron was inhibited by glucose (Fig. 3B). Taken together, these observations suggest that glucose-inhibited neurons mediate the neuroendocrine effects of 2-DG and, presumably, hypoglycemia (2,3).

Although glucose inhibits glucose-inhibited neurons through glucose metabolism, the precise details of this mechanism are still unclear and seem to differ in some

respects from mechanisms in the pancreas. For example, 2-DG clearly blocks effects of glucose on glucose-inhibited neurons (and glucose-stimulated neurons [8]) and mimics effects of hypoglycemia by stimulating glucose-inhibited neurons and by activating hypothalamus-mediated counterregulatory responses (2,3). Nevertheless, 2-DG does not generally seem to inhibit pancreatic responses to glucose and is thought not to inhibit glucokinase (16,26) (see, however, 27). This raises the possibility that some effects of glucose on glucose-inhibited (and glucose-stimulated) neurons (8) may be independent of glucokinase, although the effects of glucosamine (Fig. 4A) and other glucokinase inhibitors (6) suggest that glucokinase may at least be permissive for the effects of glucose. Thus, some observed effects of 2-DG may be indirect, possibly through hexokinase-expressing neurons that project to the recorded neurons. Similarly, in the present study, tolbutamide did not mimic the effects of glucose on glucose-inhibited neurons. In contrast, tolbutamide mimics the effects of glucose on glucose-stimulated neurons (8) and on both glucose-stimulated pancreatic  $\beta$ -cells and glucose-inhibited  $\alpha$ -cells (28). These results suggest that both glucose and tolbutamide inhibit  $\alpha$ -cells through an indirect mechanism, through stimulation of  $\beta$ -cells that in turn inhibit  $\alpha$ -cells (11). In contrast, effects of glucose on glucose-inhibited neurons (as defined in the present study) may be direct. However, the present data do not directly address this hypothesis.

Failure in the counterregulatory responses to hypoglycemia, secondary to previous hypoglycemic episodes, constitutes a major complication in type 1 diabetes (1). The present report indicates that the mechanism by which low glucose and glucopenia activate hypothalamic neurons involves an attenuation in glycolysis. It is important to note that impairments in responses to hypoglycemia would not entail impairments in glycolysis (which actually, as with 2-DG, induce a counterregulatory response), but instead counterregulatory impairments would likely entail increased glycolysis (which render the glucose signal inaccurately high, thus preventing perception of hypoglycemia). Therefore, the present studies suggest that hypoglycemia may lead to enhanced subsequent glycolysis, consistent with the increased expression of hypothalamic glucokinase subsequent to hypoglycemia (6). These data suggest that a possible strategy to reverse counterregulatory impairments could involve selective inhibition of glycolysis in glucose-sensing neurons, possibly by targeting glucokinase or the mechanisms that mediate downstream effects of glycolytic intermediates, especially NADH.

## ACKNOWLEDGMENTS

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