Original Article

Sodium-Coupled Glucose Cotransporters Contribute to Hypothalamic Glucose Sensing

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Specialized neurons within the hypothalamus have the ability to sense and respond to changes in ambient glucose concentrations. We investigated the mechanisms underlying glucose-triggered activity in glucose-excited neurons, using primary cultures of rat hypothalamic neurons monitored by fluorescence calcium imaging. We found that 35% (738 of 2,139) of the neurons were excited by increasing glucose from 3 to 15 mmol/l, but only 9% (6 of 64) of these glucose-excited neurons were activated by tolbutamide, suggesting the involvement of a ATP-sensitive K^+ channel–independent mechanism. α-Methylglucopyranoside (αMDG; 12 mmol/l), a nonmetabolizable substrate of sodium glucose cotransporters (SGLTs), mimicked the effect of high glucose in 67% of glucose-excited neurons, and both glucose- and αMDG-triggered excitation were blocked by Na^+ removal or by the SGLT inhibitor phloridzin (100 nmol/l). In the presence of 0.5 mmol/l glucose and tolbutamide, responses could also be triggered by 3.5 mmol/l αMDG, supporting a role for an SGLT-associated mechanism at low as well as high substrate concentrations. Using RT-PCR, we detected SGLT1, SGLT3a, and SGLT3b in both cultured neurons and adult rat hypothalamus. Our findings suggest a novel role for SGLTs in glucose sensing by hypothalamic glucose-excited neurons. *Diabetes* 55:3381–3386, 2006

Glucose-sensing neurons in the hypothalamus have been implicated in the control of feeding behavior and glucose homeostasis and have been the topic of recent wide interest because they may provide novel targets for the treatment of diabetes and obesity. These neurons respond to a rise in extracellular glucose levels by changing their action potential firing rate and can be simply divided into those that increase their firing rate (glucose-excited neurons) and those that decrease their firing rate (glucose-inhibited neurons) (1).

Glucose-excited neurons are thought to respond to elevated glucose levels in a manner similar to that of pancreatic β-cells, through the closure of ATP-sensitive K^+ channels (K_{ATP} channels) (2–4). This is believed to lead to membrane depolarization and the activation of voltage-dependent Ca^{2+} channels, causing Ca^{2+} influx. As in β-cells, it has been proposed that glucokinase, which is selectively expressed in brain regions containing glucose-sensing neurons (5–7), acts as the glucose sensor in hypothalamic neurons, converting the glucose signal to changes in the ATP concentration and thereby setting the level of K_{ATP} channel activity (8–10).

Although a body of evidence supports a role for metabolic signals in glucose sensing by the hypothalamus, there is also a broad consensus that K_{ATP} channel–dependent mechanisms cannot explain all of the findings and that an alternative glucose-sensing pathway must exist in some glucose-excited neurons (11). Thus, many neurons in the brain express K_{ATP} channels, yet relatively few exhibit glucose-sensing properties (12,13), and the K_{ATP} channel subunits Kir6.2 and SUR1 have been confusingly detected in both glucose-excited and -inhibited neurons (6). Furthermore, Kir6.2, SUR1, and glucokinase have only been detected in a proportion of glucose-excited neurons (6). In mice deficient in Kir6.2, glucose-excited neurons were no longer detected in the ventromedial hypothalamus (14) but were still observed in the arcuate nucleus (15). Membrane depolarization in the latter study correlated with the opening of a conductance with a reversal potential of approximately −20 mV, properties not typical of a potassium-selective (K_{ATP}) channel. ATP levels in hypothalamic glucose-excited neurons were also reported to be unchanged by elevations in extracellular glucose that increased neuronal firing (16). The data therefore support the idea that a K_{ATP} channel–independent glucose-sensing pathway operates in some glucose-excited neurons.

We have previously described a novel glucose-sensing mechanism in a glucagon-like peptide-1–secreting cell line, GLUTag, involving the activity of sodium-coupled glucose cotransporters (SGLTs) (17). These transmembrane proteins transport Na^+ and glucose concomitantly and are therefore electrogenic (18). The possibility that they may also play a role in glucose sensing in the hypothalamus is suggested by the finding that intracerebroventricular administration of an SGLT antagonist, phloridzin, enhanced food intake in rats (19) and that phloridzin inhibited glucose-induced activation of glucose-excited neurons in the ventromedial hypothalamus (20). Interestingly, it has recently been reported that human SGLT3 is not a glucose transporter, but rather a glucose sensor in the plasma membrane of cholinergic neurons, skeletal muscle, and other tissues (21).

In this study, we provide evidence that hypothalamic

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$[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; K_{ATP} channel, ATP-sensitive K^+ channel; DMEM, Dulbecco's modified Eagle's medium; αMDG, α-methylglucopyranoside; 3-O-MDG, 3-O-methyl-D-glucopyranose; SGLT, sodium-coupled glucose cotransporter.

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cells express SGLTs and that an SGLT-dependent glucose-sensing mechanism operates in some glucose-excited neurons in addition to the classical K_{ATP} channel–dependent pathway.

RESEARCH DESIGN AND METHODS

All procedures used conformed with the U.K. Animals (Scientific Procedures) Act of 1986. Primary cultures of hypothalamic neurons were prepared as described previously (16). In brief, Sprague Dawley rats, 2–4 days postnatal, were humanely killed by cervical dislocation. After decapitation, the hypothalamus was removed and transferred to a solution of HEPES-buffered saline consisting of (in mmol/l): 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 3 glucose, pH 7.3, and then finely chopped. The tissue was digested in HEPES-buffered saline supplemented with 1 mg/ml protease XIV (Sigma, Poole, Dorset, U.K.) and 1 mg/ml protease X (Sigma) for 25 min at room temperature. After digestion, the hypothalamic tissue was gently triturated using flame-polished Pasteur pipettes of decreasing diameters. The cells were pelleted by centrifugation at 1,200 rpm for 3 min and then resuspended in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% (vol/vol) fetal bovine serum (Sigma), 11 mmol/l glucose, 2 mmol/l glutamine, 5 μg/ml insulin, 10 μg/ml penicillin, and 10 μg/ml streptomycin. The cells were centrifuged as above and then resuspended in the supplemented DMEM before plating onto glass-bottomed dishes (MatTek) coated with poly-L-lysine (20 μg/ml for 1–2 h) and allowed to stick down for 1 h under an atmosphere of 5% CO_2 at 37°C. After overnight incubation in DMEM, the medium was changed to neurobasal medium (Gibco) containing 17.5 mmol/l glucose and supplemented with N2 serum (Gibco), 2 mmol/l glutamine, 10 μg/ml penicillin, and 10 μg/ml streptomycin. Cells were used 6–14 days after isolation, but similar responses were also observed in neurons cultured for 24–48 h.

Ca^{2+} measurements. A digital epifluorescence imaging system (Cairn Research, Faversham, U.K.) mounted on an inverted IX71 fluorescence microscope (Olympus, Southall, U.K.) with a 40× oil immersion objective was used to measure changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The cultured hypothalamic neurons were loaded with the Ca^{2+}-sensitive dye fura-2 AM (6 μmol/l; Molecular Probes) for 40–60 min in 3 mmol/l glucose at room temperature. Ratiometric images (340/380 nm excitation, >510 emission) were collected at 3-s intervals using Universal Imaging MetaFluor software (Cairn Research), and emission was recorded with a charge-coupled device camera (Hamamatsu Orca ER; Cairn Research). Data were expressed as changes in the fluorescence ratio. Cells were perfused with solution containing (in mmol/l): 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 3 glucose, pH 7.3, to which glucose and other substrates were added directly. Data were obtained from the soma of individual neurons. Galactose was purchased from Fisher Scientific, and all other reagents were obtained from Sigma-Aldrich (Poole, U.K.).

RT-PCR. Whole RNA was prepared from rat duodenum, rat adult hypothalamus, and cultured hypothalamic neurons using Tri reagent (Sigma). First-strand cDNA synthesis was performed for 1 h at 42°C in a total reaction volume of 25 μl containing ~1 μg RNA, 500 ng random hexamer primers (Promega), 0.5 mmol/l of each dNTP (Promega), 40 units RNAsin (Promega), 50 mmol/l Tris-HCl (pH 8.3), 75 mmol/l KCl, 10 mmol/l dithiothreitol, 3 mmol/l $MgCl_2$, and 250 units of SuperScriptII reverse transcriptase (Invitrogen). We used 1 μl of this reaction for subsequent PCR amplification performed in a volume of 50 μl containing 1 μmol of each primer of one sense/antisense pair, 0.2 mmol/l of each dNTP (Promega), 1.75 mmol/l $MgCl_2$, 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 9.0), 0.1% Triton X-100, and 1.0 units of Taq polymerase (Promega) in a PTC-200 thermocycler (MJ Research), using the following cycling protocol: initial 3-min denaturation at 94°C; followed by 25 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min; and a final elongation at 72°C for 5 min. We used 0.5 μl of this initial PCR for a secondary PCR under the same conditions with nested primer pairs. Primer pairs were designed using rat genomic sequence information from the ensemble database (from 5′ to 3′) and span exon/intron borders: SGLT1/SLC5A1 (ENSRNOG00000017775) sense TGTTACACACCCAGGGCCG, antisense GGTGAAGAGAGTACTGGCGC, nested-sense GTACTGGTGTACGGATCAGG, and nested-antisense GAGGTCAAGGAGCTCATGAG; SGLT3A/SLC5A4a (ENSRNOG00000006786) sense GAACATGTCCCACGTGAAGGC, antisense TTTACAGAAGATGGCGACCAGG, nested-sense ATGCTGTCGGTCATGTTGGC, and nested-antisense TGTCCACCTTGAGATACTTCTAC; SGLT3B/SLC5A4b (ENSRNOG00000001298) sense GAACATGTCCCACGTGAAGGC, antisense TGCAGAAGATGGCAAGCAAGAAC, nested-sense ATGCTGTCGGTCATGTTGGC, and nested-antisense GATGTAGTGGAAGAGCTGTCC; and SGLT4/SLC5A9 (ENSRNOG00000000141) sense ACCTGTCACCTCCCACGG, antisense ATATTGGAGCATCCAACTCTGGC, nested-sense ATGCCTTCCACATGCTTCGAG, and nested-antisense TGACAGATGTCAGGGTCCAC. We used 16 μl of the reaction for subsequent analysis by agarose gel electrophoresis (1.5% gel), and we visualized products by ethidium bromide fluorescence. The predicted sizes of fragments were (in bp): 323 for SGLT1, 209 for SGLT3A, 227 for SGLT3B, and 274 for SGLT4. Identity of bands was confirmed by direct sequencing.

Data analysis. All data are the means ± SE, and statistical analyses were performed using paired Student's *t* tests (two tailed, 95% CI) for comparison of means. $P < 0.05$ was considered significant. The mean 340/380 nm fluorescence ratio was calculated over a 150-s time period for each condition. Changes in the 340/380 nm ratio of ≥0.04 were counted as significant when counting numbers of cells responding to a stimulus. A smaller ratio change of ≥0.03 was used in the low substrate concentration experiments shown in Fig. 5.

RESULTS

Primary cultured rat hypothalamic neurons were loaded with fura-2 AM and monitored by fluorescence imaging with excitation wavelengths of 340 and 380 nm. Neurons were subdivided into three populations based on their responses to increasing the glucose concentration from 3 to 15 mmol/l. We found that 35% (738 of 2,139) of the neurons showed a reversible increase in the 340/380 nm fluorescence ratio of >0.04 (mean increase 0.12 ± 0.005, from a baseline ratio of 1.11 ± 0.007, $n = 738$) and were classified, on this basis, as glucose excited. We also found that 6% (127 of 2,139) of the neurons exhibited a >0.04 decrease in the ratio (mean change −0.08 ± 0.006, from a baseline ratio of 1.3 ± 0.03, $n = 127$; $P < 0.001$ vs. baseline of glucose-excited neurons) and were classified as glucose inhibited. The remainder were counted as nonresponsive. The increase in $[Ca^{2+}]_i$ in glucose-excited neurons was reversible on return to 3 mmol/l glucose and was reproducible on second application in 61% (66 of 108) of the neurons tested (Fig. 1*A* and *C*).

K_{ATP} channel closure does not account for the glucose sensitivity of all glucose-excited neurons. It has been suggested previously that glucose-excited neurons are activated in a manner similar to that of pancreatic β-cells (20), via the closure of K_{ATP} channels after glucose metabolism. To examine the effect of K_{ATP} channel closure on glucose-excited neurons, tolbutamide (100 μmol/l) was applied to the cells in perfusate containing 3 mmol/l glucose. Under these conditions only 9% (6 of 64) of neurons already designated as glucose excited showed a response to tolbutamide (Fig. 1*A*). These data suggest that activation of most glucose-excited neurons in this culture involves a mechanism distinct from K_{ATP} channel closure.

SGLT substrates trigger Ca^{2+} influx in hypothalamic neurons. To investigate whether SGLT-associated currents play a role in glucose sensing by hypothalamic glucose-excited neurons, we tested the effect of the nonmetabolizable glucose analog α-methylglucopyranoside (αMDG), which is a specific substrate of SGLTs but not of the facilitative glucose transporter (GLUT/SLC2 [solute carrier 2]) family. As shown in Fig. 1, αMDG (12 mmol/l) triggered a rise in $[Ca^{2+}]_i$ in 67% (123 of 184) of the glucose-excited neurons. Excitation of the neurons by αMDG was readily reversible on washout and reproducible on second application in 109 of 123 cells. Only 3% of neurons were inhibited by the same concentration of αMDG (Δ ratio −0.12 ± 0.02, $n = 41$), including a few glucose-excited and nonresponsive as well as glucose-inhibited neurons.

To further investigate the role of SGLTs in glucose-excited neurons, we tested the effect of the competitive SGLT inhibitor phloridzin on the responses to both αMDG and glucose (Fig. 2*A* and *C*). Application of 100 nmol/l phloridzin abolished the response to αMDG in 42 of 45

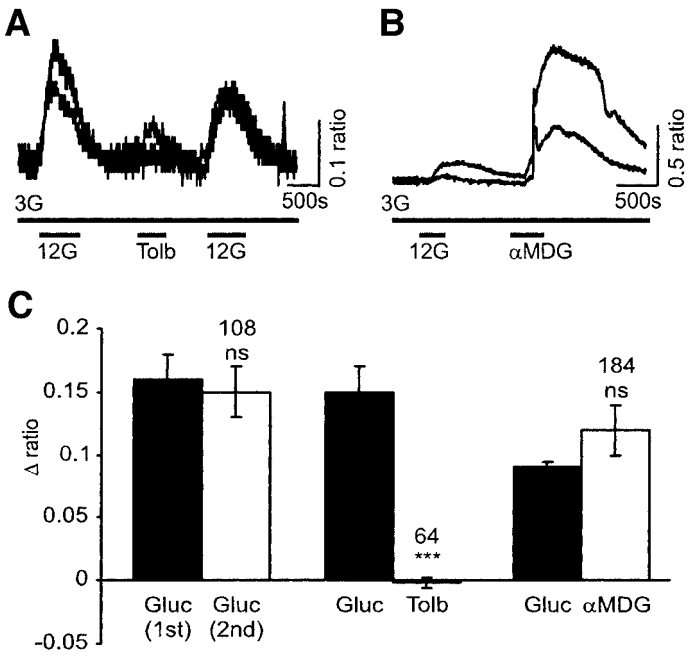

FIG. 1. Glucose-excited neurons respond to αMDG but not tolbutamide. *A*: Sample ratiometric Ca^{2+} imaging traces from primary hypothalamic neurons illustrating the increase in the 340/380 nm fluorescence ratio in response to an increase in glucose from a background level of 3 mmol/l glucose (3G) to 15 mmol/l glucose but not to 100 μmol/l tolbutamide (Tolb). *B*: Sample Ca^{2+} imaging trace showing the increase in $[Ca^{2+}]_i$ in response to the addition of 12 mmol/l glucose (12G) and 12 mmol/l αMDG to a background solution containing 3 mmol/l glucose. *C*: Histogram of the pooled data obtained as in *A* and *B*, illustrating the change in the 340/380 nm ratio (Δ ratio) in response to an increase in glucose concentration from 3 to 15 mmol/l (■) paired with responses to 12 mmol/l αMDG (□), 100 μmol/l tolbutamide (□), and a second application of glucose (12 mmol/l; □). All cells found to respond to the first increase in glucose were included in the analysis. *N* values are shown above the bars. Statistical significance was tested by comparing the mean change in ratio in response to αMDG, tolbutamide, or a second application of glucose with the first glucose response in the same cell, using a paired Student's *t* test. ***$P < 0.001$; ns, $P > 0.05$. Gluc, glucose.

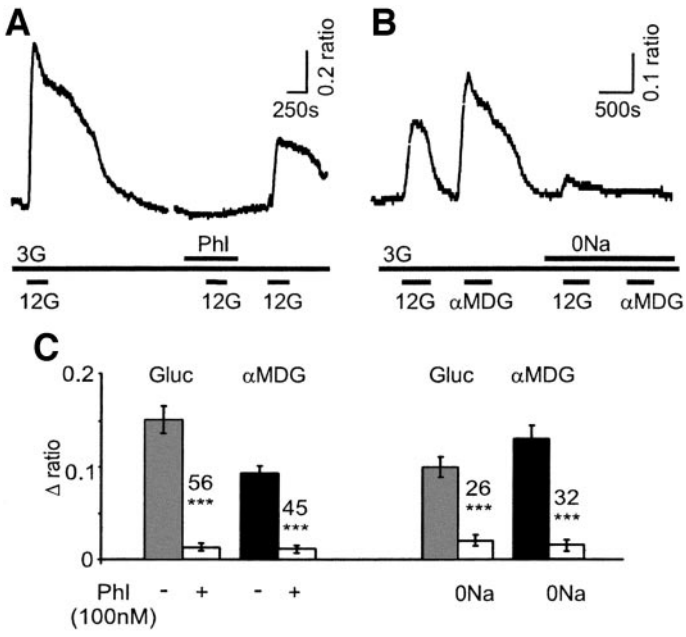

FIG. 2. Responses to glucose and αMDG are inhibited by phloridzin and removal of extracellular Na^+. *A*: Sample trace showing the responses to application of 12 mmol/l glucose (12G) under control conditions (background 3 mmol/l glucose [3G]) and in the presence of the competitive SGLT inhibitor phloridzin (100 nmol/l). *B*: Sample ratiometric trace illustrating the Ca^{2+} response to 12 mmol/l glucose and 12 mmol/l αMDG under control conditions and on removal of extracellular Na^+. *C*: Histogram of pooled data showing 12 mmol/l glucose (■) and 12 mmol/l αMDG (■)–induced Ca^{2+} responses in the absence (■) and presence (□) of 100 nmol/l phloridzin, or the presence (■) or absence (□) of Na^+. The numbers of cells are shown above the bars. Statistical significance was tested by comparing the mean substrate-triggered changes in the ratio in the absence or presence of phloridzin, or the absence or presence of Na^+, using a paired Student's *t* test. ***$P < 0.001$. Phl, phloridzin.

cells and the response to glucose in 53 of 56 cells. A higher phloridzin concentration of 200 μmol/l, which inhibits some GLUT isoforms in addition to SGLTs, had a similar effect (data not shown). Because extracellular Na^+ ions are necessary for substrate uptake by SGLTs, we also investigated the effect of replacing Na^+ with $NMDG^+$ (*N*-methyl-D-glucamine). Na^+ removal abolished the glucose response in 23 of 26 cells and the αMDG response in 30 of 32 cells (Fig. 2*B* and *C*). These data suggest a role for SGLTs in glucose sensing by cultured hypothalamic glucose-excited neurons.

Which SGLT is responsible for glucose responses in cultured hypothalamic neurons? To investigate the expression of different SGLTs in the hypothalamus, we performed RT-PCR on mRNA extracted from cultured hypothalamic neurons and adult rat hypothalamus, using primer pairs designed to amplify SGLT1, SGLT3a, SGLT3b, or SGLT4. As shown in Fig. 3, all of these SGLTs were identified in the cultured neurons, and all but SGLT4 were detected in adult hypothalamus.

SGLT1 and SGLT3 can be functionally distinguished based on their substrate specificity. Whereas glucose and αMDG are substrates for both SGLT1 and SGLT3, galactose and 3-O-methyl-D-glucopyranose (3-O-MDG) are transported by SGLT1 but not SGLT3 (21). To investigate the relative roles of SGLT1 and SGLT3 in cultured hypothalamic neurons, we therefore measured responses to galactose and 3-O-MDG (Fig. 4). Addition of galactose (12 mmol/l) or 3-O-MDG (12 mmol/l) to the 3 mmol/l glucose background triggered an increase in $[Ca^{2+}]_i$ in 24 of 65 (37%) and 18 of 40 (45%) glucose-excited neurons, respectively. Responses to both agents were impaired by phloridzin (Fig. 4*B*). Taken together, these findings suggest a role for SGLT1 in the activation of glucose-excited neurons in this preparation.

Effects of osmolarity. Because the increase in glucose concentration from 3 to 15 mmol/l would raise the osmo-

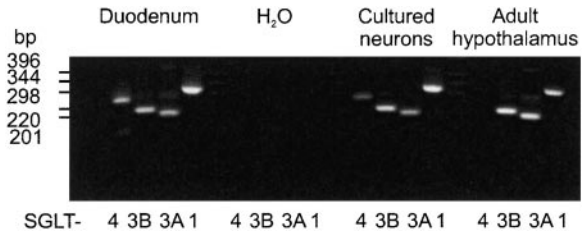

FIG. 3. Sodium-coupled glucose transporter expression in the hypothalamus. RT-PCR detects expression of SGLT1, SGLT3A, SGLT3B, and SGLT4 in primary rat hypothalamic cultures. SGLT1, SGLT3A, and SGLT3B were also readily detected in cDNA generated from a hypothalamic block from adult rat brain, whereas the SGLT4 primers only amplified a bigger (unspliced/genomic) band from this cDNA. All primer pairs did not amplify any bands when water was used instead of cDNA in the initial PCR and gave the expected band sizes when duodenal cDNA was used, for which expression of these SGLTs has been reported previously. The expected band sizes were: SGLT1 323 bp, SGLT3A 209 bp, SGLT3B 227 bp, and SGLT4 274 bp. Band identity was confirmed by direct sequencing.

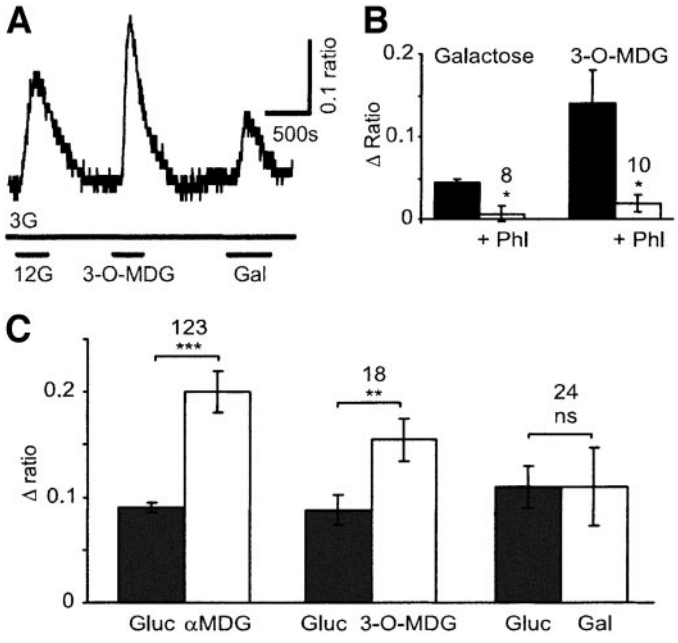

FIG. 4. SGLT1 can facilitate Ca^{2+} influx in glucose-excited neurons. *A*: Sample Ca^{2+} imaging trace from a glucose-excited neuron illustrating the response to addition of 12 mmol/l glucose (12G), 12 mmol/l 3-O-MDG, and 12 mmol/l galactose (Gal) to the control 3 mmol/l glucose (3G) solution. *B*: Histogram of pooled data showing the change in ratio triggered by 12 mmol/l galactose and 12 mmol/l 3-O-MDG in the absence (■) and presence (□) of 200 μmol/l phloridzin (Phl). The numbers of cells are shown above the bars. Statistical significance was tested by comparing the mean changes in ratio in the absence or presence of phloridzin using a paired Student's *t* test. *$P < 0.05$. *C*: Histogram illustrating that responses to 12 mmol/l αMDG (□) and 12 mmol/l 3-O-MDG (□) in glucose-excited neurons are significantly greater than the matched glucose (12 mmol/l) responses (■), whereas mean galactose (12 mmol/l; □) responses are not significantly different from 12 mmol/l glucose (Gluc). Only cells that responded to both substrates tested were included in the analysis. *N* values are shown above the bars. Statistical significance was tested by comparing the mean change in ratio in response to glucose against αMDG, 3-O-MDG, and galactose in matched cells using a paired Student's *t* test. ***$P < 0.001$; **$P < 0.01$; ns, $P > 0.05$.

larity of our solutions by ~4%, which might be sufficient to activate some hypothalamic osmoreceptors, we tested the effects of the nonabsorbed osmolyte mannitol. Mannitol (12 mmol/l) triggered a rise in $[Ca^{2+}]_i$ in 16 of 50 (32%) glucose-excited neurons, which was abolished by gadolinium (100 μmol/l), an inhibitor of osmotically sensitive channels in the hypothalamus (22) (data not shown). By contrast, the responses to elevated glucose or to αMDG, galactose, and 3-O-MDG (each at 12 mmol/l) were unaffected by prior incubation with gadolinium in most neurons. Thus, although some neurons are responsive to changes in osmolarity over this concentration range, this does not account for the actions of the SGLT substrates.

SGLT operates at low- as well as high-glucose concentrations. Because glucose concentrations within the brain are estimated to be approximately one-third of the corresponding plasma levels, we tested whether SGLT-associated currents could also be detected at lower substrate concentrations (Fig. 5). Decreasing the glucose level from 3 to 0.5 mmol/l was associated with a reduction in $[Ca^{2+}]_i$ in 20 of 69 neurons. Within this subpopulation, which should include glucose-excited neurons sensitive to lower glucose levels, 5 of 20 cells exhibited an elevation of $[Ca^{2+}]_i$ in response to both 100 μmol/l tolbutamide (mean Δ ratio +0.05 ± 0.01) and the subsequent addition of 3.5 mmol/l αMDG (mean Δ ratio +0.04 ± 0.01, $P < 0.01$ vs.

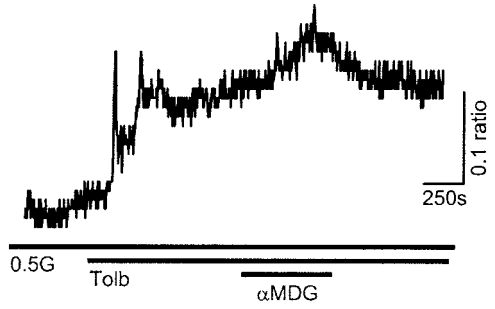

FIG. 5. αMDG triggers Ca^{2+} elevations at low substrate concentrations. The imaging trace shows an example of a glucose-excited neuron inhibited by a decrease in glucose from 3 to 0.5 mmol/l, which was activated by 100 μmol/l tolbutamide (Tolb) and further activated by 3.5 mmol/l αMDG. 0.5G, 0.5 mmol/l glucose.

background in tolbutamide, by one-sample Student's *t* test). These data suggest that both K_{ATP} channel– and SGLT-dependent pathways underlie the responses of glucose-excited neurons at lower glucose concentration ranges.

Inhibitory effect of glucose metabolism. During the course of these experiments, we noticed that the $[Ca^{2+}]_i$ changes triggered by addition of the nonmetabolizable substrates αMDG (12 mmol/l) and 3-O-MDG (12 mmol/l) were consistently larger than those triggered by galactose (12 mmol/l) or increasing the glucose concentration to 15 mmol/l in the same cells. These differences are evident in Figs. 1*B* and 4, which only include cells that were tested with and responded to both agents, to reduce the effect of interexperiment variation. This result cannot be accounted for by the properties of SGLT1 because this transporter does not distinguish between glucose and αMDG when studied in heterologous expression systems (21). The findings therefore raise the possibility that the metabolizable sugars glucose and galactose, unlike αMDG and 3-O-MDG, might have both inhibitory and stimulatory actions within the same cell. To investigate further the idea that metabolism impairs the response to SGLT substrates, we measured the response to αMDG in the presence and absence of an alternative metabolite, lactate (all in the presence of 3 mmol/l glucose and 100 μmol/l gadolinium). As shown in Fig. 6, αMDG triggered smaller changes in the fura-2 ratio when added in the presence of 10 mmol/l lactate.

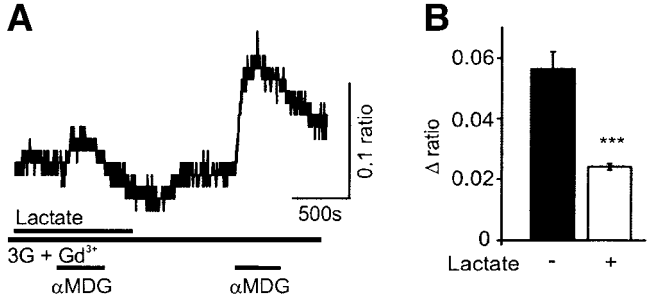

FIG. 6. Lactate reduces the magnitude of αMDG responses. *A*: Representative Ca^{2+} imaging trace illustrating that responses to 12 mmol/l αMDG are reduced in the presence of 10 mmol/l lactate. Gadolinium (100 μmol/l) was present throughout the experiment. *B*: Histogram of pooled data showing the magnitude of αMDG-triggered responses in the presence (□) and absence (■) of 10 mmol/l lactate ($n = 32$). Statistical significance was tested by comparing αMDG responses in the absence or presence of lactate, using a paired Student's *t* test. ***$P < 0.001$.

DISCUSSION

Using calcium imaging as a marker of neuronal excitability, we detected glucose-excited, glucose-inhibited, and nonresponsive neurons in primary hypothalamic cell cultures, in proportions similar to those described previously (1,23). Thus, ~30% of neurons were excited by raising the glucose concentration from 3 to 15 mmol/l, ~6% were inhibited, and the remainder were nonresponsive over the same concentration range. The mechanism of glucose sensing in the glucose-excited neurons could not be attributed solely to K_{ATP} channel closure because tolbutamide only increased intracellular Ca^{2+} in ~10% of glucose-excited neurons in the presence of 3 mmol/l glucose. Furthermore, the nonmetabolizable sugars αMDG and 3-O-MDG mimicked the action of glucose in glucose-excited neurons, indicating that metabolic generation of ATP is not a prerequisite for the sensing of glucose analogs. The sensitivity of the glucose-sensing machinery to αMDG, its dependence on extracellular Na^+, and its inhibition by low concentrations of the SGLT inhibitor phloridzin suggest that interaction of the sugars with SGLTs is a critical step in glucose recognition.

The idea that K_{ATP} channel–independent mechanisms play a role in glucose sensing in glucose-excited neurons has been suggested previously, since it has been reported that glucose-excited neurons can also be detected in arcuate nucleus of Kir6.2-deficient mice (15) and that glucose application activates a conductance that may represent nonselective cation channel activity, based on its reversal potential of approximately −20 mV. Raising the glucose concentration from 3 to 15 mmol/l has also been reported to be relatively ineffective at increasing the cytoplasmic ATP concentration in cultured hypothalamic neurons (16).

SGLTs classically function as Na^+-dependent transporters, coupling the uptake of each sugar molecule to the influx of a fixed number of Na^+ ions (usually one to two), and thereby using the downhill Na^+ gradient to drive sugar uptake against its concentration gradient (18). They are best known for their roles in absorbing glucose from the lumen of the intestine and kidney tubules. Because Na^+ influx via SGLTs is not directly coupled to the movement of a counter ion, the Na^+ flux generates a small inward current whose magnitude is directly determined by the sugar concentration. In the cell line GLUTag, we showed that this SGLT-associated current, though only a few picoamperes in magnitude, is large enough to trigger membrane depolarization (17). More recent work has shown that human SGLT3 has lost its transporter activity and functions as a glucose-dependent Na^+ channel (24). Regardless of whether they act primarily as sugar-dependent coupled transporters or ion channels, the reported glucose K_m values of SGLT1 (~0.2 mmol/l, when expressed heterologously in *Xenopus* oocytes) and SGLT3 (~6 mmol/l in *Xenopus* oocytes) make them ideally suited to the role of sugar sensors over the physiological glucose range (21). Because SGLTs are able to concentrate glucose in the cytoplasm, their activity at lower glucose levels might also function to increase the cytoplasmic glucose concentration to levels more within the reported operational range of glucokinase.

In vitro studies have shown that SGLT1 and SGLT3 both transport glucose and αMDG, but that only the former transports 3-O-MDG and galactose (21). The responsiveness of many primary cultured neurons to 3-O-MDG and galactose therefore suggests a role for SGLT1 in these cells. However, because a higher proportion of glucose-excited neurons were activated by αMDG (67%) than by 3-O-MDG (45%, $P < 0.01$ by χ^2 test), SGLT3 might also play a role in some cells. Using RT-PCR, we identified SGLT1, SGLT3A, SGLT3B, and SGLT4 in cultured neurons but only SGLT1, SGLT3A, and SGLT3B in adult rat hypothalamus. The finding that 45% of glucose-excited neurons were activated by SGLT1-specific substrates compares with a previous report that at least 25% of glucose-excited neurons expressed SGLT1, as measured by single-cell RT-PCR (6). Recent evidence points to a role for glial cells in hypothalamic glucose sensing (16,25), raising the possibility that SGLTs might be involved in glucose uptake into glial cells, with subsequent passage of metabolic substrates to the neurons. However, it has been shown previously that expression of SGLT1 and SGLT3 (also known as SAAT1) was restricted to the neuronal population in cultures of glial cells and neurons from whole embryonic rat brain (26).

Although both metabolizable and nonmetabolizable sugars triggered elevations of intracellular $[Ca^{2+}]_i$, the magnitude of the response was influenced by whether the sugar could be metabolized. Thus, glucose and galactose tended to trigger smaller Ca^{2+} increments than αMDG and 3-O-MDG in the same cells. These data suggest that metabolism can exert an inhibitory effect on the Ca^{2+} response, an idea supported by the finding that addition of lactate reduced the magnitude of responses to αMDG.

The glucose levels to which hypothalamic glucose-sensing neurons are exposed in vivo remain controversial. When extracellular glucose levels in the brain were measured at different plasma glucose concentrations, it was found that brain glucose varied between 1 and 2.5 mmol/l when the plasma glucose was altered from 5 to 8 mmol/l (27) and that at plasma glucose levels of 15–17 mmol/l, brain concentrations were ~4.5 mmol/l (28). Most central neurons probably therefore experience glucose concentrations that are approximately one-third of those in the plasma, but it has been argued that areas of the hypothalamus where the blood-brain barrier is deficient might be exposed to levels closer to those measured in the plasma. Previous studies on hypothalamic glucose-sensing mechanisms have been carried out at both low (<5 mmol/l) and high (5–15 mmol/l) glucose ranges. At low glucose concentrations, there is strong evidence for glucokinase-dependent K_{ATP} channel closure as a mechanism of glucose sensing (10), whereas at higher concentrations evidence from several studies favors the involvement of alternative glucose-sensing pathways (15,16). Although the SGLT-dependent pathway dominated at higher glucose levels in our experiments, we found additive effects of K_{ATP} channel closure and SGLT activity at the lower substrate range.

In conclusion, our findings suggest that the glucose-dependent activity of SGLTs can operate as a glucose-sensing mechanism in some hypothalamic glucose-excited neurons. Because currents associated with sugar transport by SGLTs are very small (17), the effectiveness of this pathway would be modulated by the input resistance of the cell, and hence by the rate of metabolism and consequent activity of K_{ATP} and other ion channels. The glucose-dependent activity of SGLTs at low as well as high substrate concentrations makes this an ideal glucose-sensing mechanism over a range of physiological glucose levels. Further studies are now required to determine the

relative roles of SGLT and metabolic K_{ATP} channel closure in different populations of glucose-excited neurons.

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