Physiological and Molecular Characteristics of Rat Hypothalamic Ventromedial Nucleus Glucosensing Neurons

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To evaluate potential mechanisms for neuronal glucosensing, fura-2 Ca^{2+} imaging and single-cell RT-PCR were carried out in dissociated ventromedial hypothalamic nucleus (VMN) neurons. Glucose-excited (GE) neurons increased and glucose-inhibited (GI) neurons decreased intracellular Ca^{2+} ($[Ca^{2+}]_i$) oscillations as glucose increased from 0.5 to 2.5 mmol/l. The Kir6.2 subunit mRNA of the ATP-sensitive K^+ channel was expressed in 42% of GE and GI neurons, but only 15% of nonglucosensing (NG) neurons. Glucokinase (GK), the putative glucosensing gatekeeper, was expressed in 64% of GE, 43% of GI, but only 8% of NG neurons and the GK inhibitor alloxan altered $[Ca^{2+}]_i$ oscillations in ~75% of GK-expressing GE and GI neurons. Insulin receptor and GLUT4 mRNAs were coexpressed in 75% of GE, 60% of GI, and 40% of NG neurons, although there were no statistically significant intergroup differences. Hexokinase-I, GLUT3, and lactate dehydrogenase-A and -B were ubiquitous, whereas GLUT2, monocarboxylate transporters-1 and -2, and leptin receptor and GAD mRNAs were expressed less frequently and without apparent relationship to glucosensing capacity. Thus, although GK may mediate glucosensing in up to 60% of VMN neurons, other regulatory mechanisms are likely to control glucosensing in the remaining ones. *Diabetes* 53:549–559, 2004

Unlike most neurons in the brain, glucosensing neurons utilize glucose as a signaling molecule to alter their firing rate as a means of sensing and regulating glucose metabolism in the body (1). Glucose-excited (GE) neurons increase, whereas glucose-inhibited (GI) neurons decrease their firing rate as ambient glucose levels rise (2–4). Although these neurons can respond directly or indirectly to either the complete absence of glucose or to levels as high as 20 mmol/l (2,5–9), it is likely that their primary range is 0.5–3.5 mmol/l glucose under physiological conditions (4,10–12). As in the pancreatic β-cell (13), the ATP-sensitive K^+ (K_{ATP}) channel is an important component of glucosensing in GE neurons (3,5,14). The K_{ATP} channel is an octameric protein consisting of a pore-forming, inwardly rectifying K^+ channel (Kir6.1 or Kir6.2) and a sulfonylurea receptor (SUR-1 and -2) subunit (15–18). In the β-cell, the K_{ATP} channel is composed of Kir6.2 and SUR-1 (15–18). Although the K_{ATP} channel may well be required for glucosensing in ventromedial hypothalamic GE neurons (14), the mere presence of the channel does not necessarily ensure that a given neuron will be glucosensing, as this channel is widely expressed throughout the brain (10,19). Furthermore, GI neurons clearly do not use the K_{ATP} channel to sense glucose (4). Thus, another regulatory step is likely to be critical for neuronal glucosensing.

The control of both glycolysis (20,21) and glucose transport (22) have been proposed as regulators of β-cell glucosensing. In the pancreas, glucokinase (GK) (hexokinase IV, ATP: D-glucose 6-phosphotransferase) regulates glycolytic flux and intracellular ATP production in both β- and α-cells (23) and is a primary regulator of ATP production, K_{ATP} channel activity, and insulin secretion in β-cells (24). GK-mediated ATP production raises the ATP-to-ADP ratio and inactivates the K_{ATP} channel. The increased ATP-to-ADP ratio leads to membrane depolarization, Ca^{2+} influx, and increased insulin release in the β-cell (24) and increased firing rate in glucosensing neurons (3–5,8,9,24). Most neurons express hexokinase-I (HK-I), which is unlikely to regulate the rate of glycolysis because it is both saturated at physiological brain glucose levels and inhibited by its product, glucose-6-phosphate (25,26). For this reason, GE neurons might use GK as a regulatory step in glucosensing (5,8,10,27). Inhibition of GK activity decreases intracellular Ca^{2+} ($[Ca^{2+}]_i$) oscillations in dissociated hypothalamic GE neurons and decreases firing of GE neurons in hypothalamic slices (5,8,10,27). On the other hand, GK inhibition increases $[Ca^{2+}]_i$ oscillations in GI neurons (10). However, it is unclear how GK, with a K_m for glucose phosphorylation of 8–10 mmol/l (28–31), might regulate neuronal glucosensing when physiological brain glucose levels are in the 0.5–3.5 mmol/l range (11,12). One possibility is that the GK regulatory protein (GKRP),

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AUC, area under the curve; $[Ca^{2+}]_i$, intracellular Ca^{2+}; GABA, γ-aminobutyric acid; GE, glucose excited; GI, glucose inhibited; GK, glucokinase; GKRP, GK regulatory protein; HK-I, hexokinase-I; INS-R, insulin receptor; K_{ATP} channel, ATP-sensitive K^+ channel; Kir, pore-forming, inwardly rectifying K^+ channel; LDH, lactate dehydrogenase; Lepr-b, leptin receptor b; MCT, monocarboxylate transporter; mPCR, multiplex PCR; NG, nonglucosensing; sc-RT-PCR; single-cell RT-PCR; SGLT-1, Na^+-D-glucose cotransporter-1; SUR, sulfonylurea receptor; VMN, ventromedial nucleus.

TABLE 1
Criteria for defining change in $[Ca^{2+}]_i$ in glucose-excited and glucose-inhibited neurons

Cell type	Treatment (mmol/l)	Latency (min; means ± SD)	Standard latency (min; means ± 1.5 SD)	Percentage change in $[Ca^{2+}]_i$ AUC (mean % ± SD)	Minimum change in $[Ca^{2+}]_i$ AUC (mean % − 1.5 SD)
Glucose-excited neurons	2.5 G→0.5 G	1.8 ± 1.6	4.2	56 ± 24 ↓	20% ↓
	0.5 G→2.5 G	2.9 ± 1.8	5.6	63 ± 20 ↑	33% ↑
	2.5 G→+4 alloxan	2.9 ± 2.0	5.9	58 ± 26 ↓	20% ↓
Glucose-inhibited neurons	2.5 G→0.5 G	2.8 ± 2.3	6.3	79 ± 13 ↑	59% ↑
	0.5 G→2.5 G	2.8 ± 2.2	6.1	75 ± 19 ↓	46% ↓
	2.5 G→+4 alloxan	2.8 ± 2.0	5.8	73 ± 17 ↑	47% ↑

Dissociated VMN neurons from 14- to 21-day-old rat pups were subjected to changing glucose (G) concentrations from 2.5 to 0.5 or 0.5 to 2.5 mmol/l in the presence or absence of 4 mmol/l alloxan. Latencies and changes in $[Ca^{2+}]_i$ responses (AUC) were determined from the Ca^{2+} traces of a total of 108 individual glucose-excited or -inhibited and nonglucosensing neurons as described in RESEARCH DESIGN AND METHODS and illustrated in Fig. 1. Direction of arrows indicates decreased (↓) or increased (↑) $[Ca^{2+}]_i$.

which inhibits GK activity in the liver and is present in the brain (32), might possibly lower the functional K_m of GK.

GLUT2 has also been proposed as a regulator of β-cell glucosensing (22). Although GLUT2 is expressed in the brain (3,13,22,28,33), it appears to be located predominantly in astrocytes (34). Most neurons express primarily GLUT3, a high-capacity, low-K_m transporter that is largely saturated at physiological brain glucose levels (35). On the other hand, the insulin-sensitive GLUT4 (36) is expressed in neurons and has a K_m within the physiological range for brain glucose (36). Also, GLUT4 and insulin receptor (INS-R) expression overlap in brain areas involved in glucosensing (37) and thus might play a potential role in neuronal glucosensing. Finally, the high-affinity Na^+-D-glucose cotransporter-1 (SGLT-1) is expressed in brain (38) and its inhibition with phloridzin reduces activity in hypothalamic GE neurons (8). However, SGLT-1 has not been localized definitively on glucosensing neurons. Thus, the type of GLUT present and its potential role in neuronal glucosensing remains to be determined. Finally, the activity of glucosensing neurons is affected by both metabolites (i.e., lactate, ketone bodies) (39,40) and peripheral hormones (e.g., insulin, leptin) that circulate in proportion to the amount of stored adipose tissue (41). Lactate, produced largely by astrocytes (42), and ketone bodies from the periphery can serve as alternate energy sources or signaling molecules to glucose once it is transported into neurons by monocarboxylate transporters (MCTs) (43). Current evidence suggests that neurons express mainly the MCT-2 isoform (44).

Given the fact that glucosensing neurons respond to such a wide range of metabolic and hormonal signals relating to the status and control of energy homeostasis, the term "metabolic sensing" neurons might be more apt (45). However, no studies have documented the actual molecular characteristics by which glucosensing neurons sense and respond to this wide range of signals. For this reason, we used Ca^{2+} imaging combined with single-cell RT-PCR (sc-RT-PCR) to identify the mRNA species expressed in individual GE, GI, and nonglucosensing (NG) dissociated neurons from the hypothalamic ventromedial nucleus (VMN) of 14- to 21-day-old rat pups.

RESEARCH DESIGN AND METHODS

All work was approved by the institutional animal care and use committee of the East Orange Veterans Affairs Medical Center. Outbred male 14- to 21-day-old pups were housed individually on a 12:12-h light:dark schedule (lights on at 0800) and kept at 22–23°C. Food (Purina rat chow #5001) and water were available ad libitum to dams and pups.

Calcium imaging of glucosensing neurons. As previously described (10), the VMN was punched out of slices made through the ventrobasal hypothalamus. Single VMN neurons were dissociated by papain digestion and trituration and then maintained in a HEPES-buffered balanced salt solution. Fluorescent imaging measurement of $[Ca^{2+}]_i$ was carried out using fura-2 (10). The values for R_{min}, R_{max}, and constant β (the 380-nm wavelength in the Ca^{2+}-free and Ca^{2+}-bound states) were calculated from measurements with fura-2–free acid in solution in the microscope chamber. The dissociation constant (K_d) for the fura-2-Ca^{2+} complex was taken as 224 nmol/l (46).

Criteria for quantitating changes in $[Ca^{2+}]_i$. All experiments began with neurons held at 2.5 mmol/l glucose. Changes in glucose concentration and/or the addition of drugs (4 mmol/l alloxan, 200 μmol/l tolbutamide) were maintained for 10–15 min after administration. Criteria for a significant change in $[Ca^{2+}]_i$ from one condition to another were first determined from the recordings of 108 neurons and their subjective classification as GE, GI, or NG. Then latency to onset of change in $[Ca^{2+}]_i$ oscillations was determined subjectively. From these measures, latencies for each neuronal type and change in condition were defined conservatively as the standard latency (mean latency to onset + 1.5 SD) (Table 1). Next, the integrated area under the curve (AUC) for changes in $[Ca^{2+}]_i$ oscillations was calculated for changes occurring over the 7-min period after the mean latency for each neuronal type and condition. Both AUC and interpolation of the baseline were determined using Origin 7.0 software (OriginLab). This 7-min period was chosen as a standard time as cells were examined for different durations. Minimal changes in the AUC during this 7-min period were defined as the mean − 1.5 SD (Table 1). All neurons were evaluated by these criteria and classified as GE, GI, or NG (Table 1). GE neurons decreased their AUC for $[Ca^{2+}]_i$ fluctuations after a decrease in glucose from 2.5 to 0.5 mmol/l and increased fluctuations after an increase in glucose from 0.5 to 2.5 mmol/l and in response to the sulfonylurea tolbutamide (200 μmol/l) (10) (Fig. 1*A*). GI neurons increased their $[Ca^{2+}]_i$ fluctuations when glucose was decreased from 2.5 to 0.5 mmol/l and decreased fluctuations when glucose was increased from 0.5 to 2.5 mmol/l (10). Neurons not meeting standard latencies and/or the minimal AUC criteria for GE or GI neurons were classified as NG. Subsets of neurons were also categorized by their response to alloxan (Table 1), a GK inhibitor (47,48) that alters glucose-evoked Ca^{2+} response in both GE and GI neurons with an effective concentration of ~4 mmol/l, which is not toxic to neurons (10).

sc-RT-PCR. After characterization by Ca^{2+} imaging, cytoplasmic mRNA of individually imaged cells was analyzed by sc-RT-PCR. Cytoplasm from each neuron was aspirated into a micropipette that was prefilled with diethyl pyrocarbonate–treated water containing 1 μl RN_{ASE} OUT RNase inhibitor (Invitrogen, Carlsbad, CA). The micropipette contents were expelled into a microtube where cDNA synthesis was performed with a Superscript II first-strand synthesis kit (Invitrogen) following the manufacturer's directions. The RT reaction was incubated at 42°C for 50 min after heat inactivation at 70°C. cDNA was purified to completely remove inhibitory RT components by slight modification of the methods described by Liss (49). Briefly, 1 μg glycogen, 250 ng polyC RNA, 250 ng polydC DNA, and a 1/10 volume of 2 mol/l sodium acetate (pH 5.5) were added to the single-cell cDNA reactions. DNA was precipitated overnight with a 3.5 vol of ethanol at −20°C. After a 60-min centrifugation at 4°C (13,000*g*), the pellet was washed with 100 μl of 75% ethanol and pelleted again by centrifugation for 30 min. The cDNA pellet was then dried and dissolved in 20 μl of PCR-grade water.

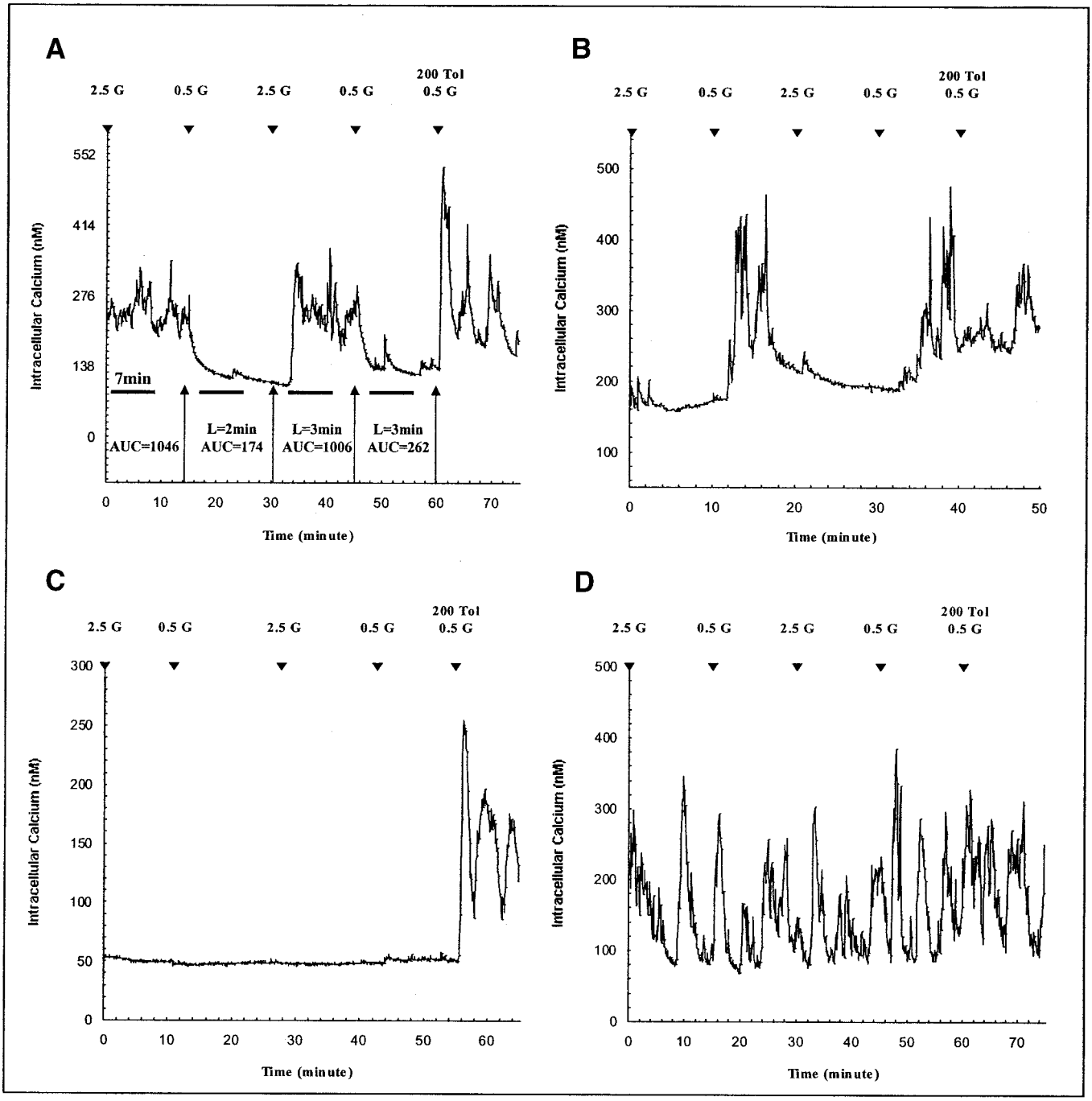

FIG. 1. Representative $[Ca^{2+}]_i$ responses used to assess the glucosensing properties of dissociated VMN neurons. Neurons were dissociated from VMN punches from 14- to 21-day-old rats, loaded with fura-2/AM. Their glucosensing type was identified using the criteria outlined in RESEARCH DESIGN AND METHODS and Table 1. *A*: Assessment of a GE neuron after changes in medium glucose concentrations or the addition of 200 μmol/l tolbutamide (Tol; *long arrows*). The horizontal bars show the 7-min periods of the Ca^{2+} trace used to calculate the AUC after the mean latency (L) from Table 1. GE neurons are where changing glucose (G) from 2.5 to 0.5 mmol/l suppressed $[Ca^{2+}]_i$ oscillations, whereas increasing glucose from 0.5 to 2.5 mmol/l or adding 200 μmol/l tolbutamide at 0.5 mmol/l increased $[Ca^{2+}]_i$ oscillations. *B*: GI neuron with $[Ca^{2+}]_i$ oscillations at 0.5 mmol/l, but none at 2.5 mmol/l glucose or with 200 μmol/l tolbutamide. *C* and *D*: NG neurons unaffected by changes in glucose concentrations but responsive to tolbutamide in *C*.

After the purification of single-cell cDNA, the PCR was performed using specific primers (Table 2). The glial fibrillary acidic protein (50) was used to exclude astrocytes and β-actin was used as an internal control for constitutively expressed mRNA. Primer sequences were designed using Biology WorkBench Primer design software. Where feasible, intron sequences were included in the amplicon to distinguish genomic DNA from mRNA. Amplification was carried out in a LightCycler (Roche PerkinElmer, Foster City, CA) using 40 cycles (95°C, 1 s; 56–63°C, 2 s; 68°C, 30 s) with Advantage 2 Polymerase Mix (BD Biosciences, Palo Alto, CA) following the manufacturer's description. The annealing temperature and Mg^{2+} concentration were varied for each set of primers to obtain optimal amplification. Amplified products were run directly on a 1.5% agarose gel and visualized by ethidium bromide staining. Gels were imaged and photo inverted for presentation. Each 10-μl reaction volume contained 1/10 volume of cDNA as template.

To optimize conditions for primer amplification and standardize for the linearity of the amplification process, hypothalamic cDNA was used. Hypothalamic cDNA was generated from total RNA that was extracted from rat hypothalamic tissue using a Purescript RNA isolation kit (Gentra Systems) and tissues were treated with DNA-free DNAse (Ambion, Austin, TX). For both single and multiplex RT-PCR (see below), single species of RNA were

TABLE 2
Sequences of each primer pair and their location in sequences cited in GenBank

	Sense primer	Antisense primer	Amplicon size (bp)	GenBank accession no.
Conventional RT-PCR primers				
HK-I	CAACAGTGTGAAGTCGGCCT	CTCTGTCAAACTCGGTTCGG	175	NM_012734
GLUT-3	TGTTCGCTGTTACTGTTGCC	CACACATAACGACCAGAGCG	170	NM_017102
MCT-1	ATGTTGTCCTGTCCTCCTGG	CTCCGCTTTCTGTTCTTTGG	172	D63834
MCT-2	TTCTTGCTGTCTGTGATGGC	AACACGACTATCCCACTGGC	395	X97445
LDH-A	GGCAACCCTTAAGGAAAAGC	CTTCCAAGACATCCACCAGG	314	NM_012595
LDH-B	TAAGGAAGAACAGGTCCCCC	TGAAGATGTTCACGTTTCGC	167	NM_017025
Lepr-b	CCATTCCCAGCTCACTGTCT	GAACAGGATTGAAACTGGGG	137	D84551
GAD	CTGCTTCCAGCTAAGAACGG	GGATCTGCTCCAGAGACTCG	198	NM_017007
GFAP	CCATGGTCCTACCAGACACC	ACACAGCCAGGTTGTTCTCC	443	L27219
β-Actin	CCGTAAAGACCTCTATGCCA	AAGAAAGGGAAAAGCCA	300	V01217
Multiplex RT-PCR primers for first and second amplifications				
GK				
1st	CCTGAAGAAGGTGATGAGCC	AGGGAAGGAGAAGGTGAAGC	373	
2nd	GATGCAGAAGGAGATGGACC	GGTTCCTCCCAGGTCTAAGG	139	NM_012565
GKRP				
1st	GTCGTCATAGGCATCTCTGTGG	CTGGTTGGTGAGTTCATCCTTC	624	
2nd	ATCCTGAGGACATTTGAGCG	TGTGGATGCACTCTACTCCG	172	NM_013120
GLUT2				
1st	AAGGATCAAAGCCATGTTGG	GGAGACCTTCTGCTCAGTCG	502	
2nd	TGCTGGAAGAAGCGTATCAG	GGCCAAGTAGGATGTGCCAG	206	NM_012879
GLUT4				
1st	TCTTTGAGATTGGTCCTGGC	TACTGGGTTTCACCTCCTGC	312	
2nd	GGTTTCTCCAACTGGACCTGTAA	ACGGCAAATAGAAGGAAGA	95	NM_012751
SGLT-1				
1st	CGGGCAGCTCTTTGACTACATCCA	AAGGCGGGGTTCAGGCAAAATA	661	
2nd	CTTCTGGGGACTGATTCTCG	CGCTCTTCTGTGCTGTTACG	255	D16101
Kir6.2				
1st	CGTCACAAGCATCCACTCCT	CACCTGCATATGAATGGTGG	328	
2nd	CCTTTTCTCCATCGAGGTCC	TGAAGATGAGGGTTTCTGCC	188	X97041
Kir6.1				
1st	AGCCACTGACCTTGTCAACC	GGAGTCATGAATTGCACCTT	402	
2nd	CATAGTGATTCTCGAGGGCG	AAGGTTTCTCGTCCAGCTCC	212	D42145
SUR-1				
1st	TGGACCCAGAGAAGAAATGC	ACAAAGGAGGCAAAGACGC	506	
2nd	TGAAGCAACTGCCTCCATC	GAAGCTTTTCCGGCTTGTCC	182	L40624
SUR-2				
1st	AGTGGCAACAACCTGAAACC	AATGTGGCTAAACCACTGCC	377	
2nd	TTCTAACCTATCCATGGGCG	TTCTAACCTATCCATGGGCG	218	D83598
INS-R				
1st	CTGAAGGAGCTGGAGGAGTC	GATTTCATGGGTCACAGGGC	426	
2nd	GCCACTACACCCACACTTCC	CTTAGCTTCAGGCATGGTCC	233	M29014

GFAP, glial fibrillary acidic protein.

amplified only within the linear portion of the curve generated from hypothalamic tissue. Hypothalamic RNA without DNAse treatment and RT was used to determine the size of any PCR products that resulted from genomic DNA amplification.

RT multiplex PCR with nested primers. Some mRNA species were present in very low abundance and required the use of additional steps for detection. For these, a second round of amplification was carried out with nested primers by multiplex PCR (mPCR) using the first-round PCR products as a template (Table 2) and a shorter extension time (51). The second-round reaction contained 1 μl of first-round PCR product as a template. The mRNAs examined by RT-mPCR included GK, GLUT2, GLUT4, SGLT-1, GKRP, Kir6.2 and Kir6.1, SUR-1 and -2, and INS-R. Serially diluted hypothalamic cDNA was used to optimize PCR conditions that produced reliable amplification with maximum sensitivity (Fig. 2*A* and *C*). The predicted sizes (bp) of the mPCR-generated fragments are given in Table 2.

Statistics. Data were analyzed using the χ^2 test for nonparametric statistics. Significant intergroup differences were defined at the $P = 0.05$ level.

RESULTS

$[Ca^{2+}]_i$ oscillation responses to altered glucose concentrations. Overall, we assessed the physiological and molecular properties of >300 acutely dissociated VMN neurons by combining Ca^{2+} imaging with sc-RT-PCR. To examine glucosensing under physiologically relevant metabolic conditions for VMN neurons (4), activity by calcium imaging was recorded at different extracellular glucose concentrations between 0.5 and 2.5 mmol/l (Fig. 1). In general, GE neurons had slightly faster responses and smaller $[Ca^{2+}]_i$ oscillation responses than GI neurons after alterations in ambient glucose levels (Table 1). NG neurons were of two types: most (77%) were inactive at either

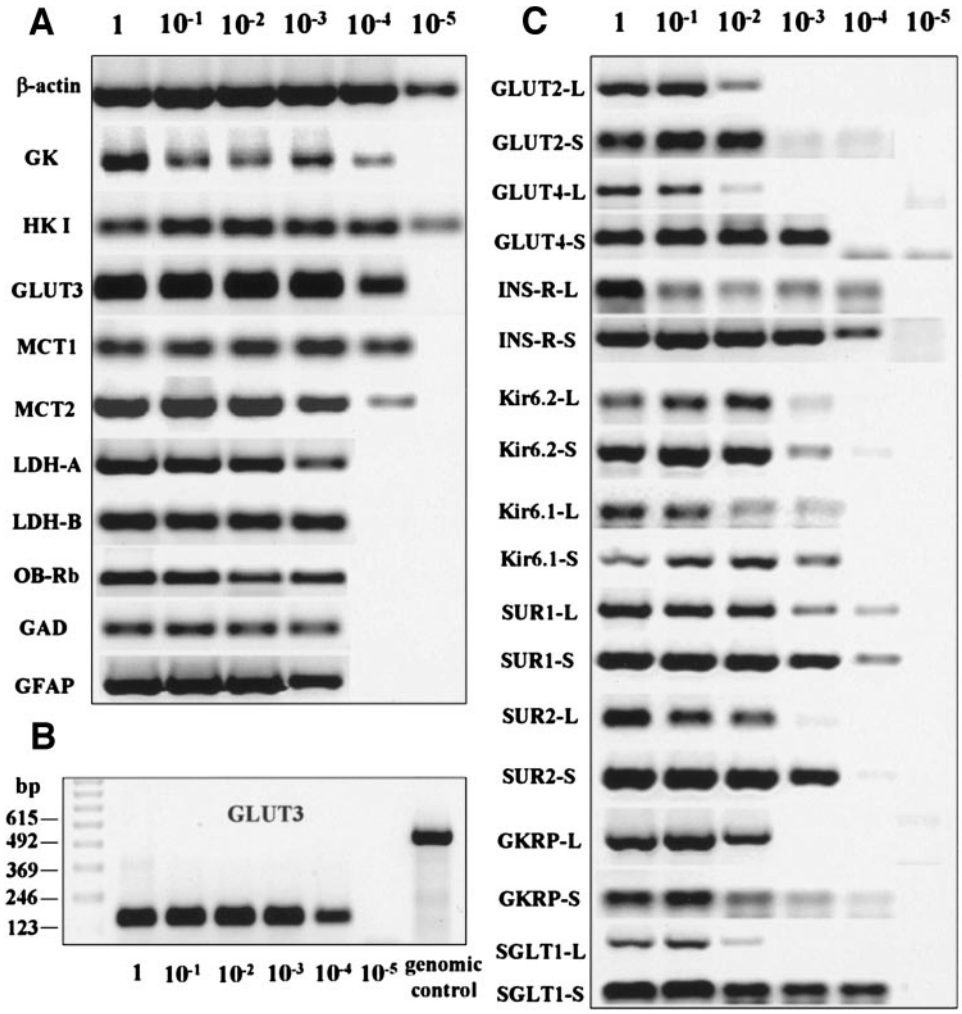

FIG. 2. Sensitivity and specificity of the sc-RT-PCR procedure. *A*: A 1.5% agarose gel showing the sensitivity of primer pairs. PCR amplification was performed on serially diluted (undiluted to 10^5-fold diluted) hypothalamic cDNA from 14- to 21-day-old rats. Each amplification generated an amplicon of the size (bp) predicted by its mRNA sequence (Table 2). *B*: Agarose gel of a genomic control for the GLUT3 primer generated without DNAse treatment and RT in the reaction. Here genomic DNA can be distinguished by its larger size, including the intron sequence. *C*: Agarose gel showing the sensitivity of primer pairs for first- (long [L]) and second-round (nested PCR primers; short [S]) of multiplex PCR amplifications in serial dilutions of hypothalamic cDNA. As expected, mPCR improved sensitivity.

0.5 or 2.5 mmol/l glucose (Fig. 1*C*), whereas 23% showed continuous, spontaneous $[Ca^{2+}]_i$ oscillations (Fig. 1*D*). Neither type altered its $[Ca^{2+}]_i$ oscillations when glucose concentrations changed. Of all the neurons tested for sc-RT-PCR, 32% were GE, 22% were GI, and the rest (46%) were NG.

sc-RT-PCR analysis of various mRNA species in GE, GI, and NG neurons. The majority of mRNA species of interest could be identified by a single round of amplification using conventional RT-PCR on the contents of single cells (Table 3; Figs. 2–4). In all these cases, the number of rounds of amplification fell within the linear range of the standard curve from pooled hypothalamic dilutions (Fig. 2*A* and *C*). However, several species were in very low abundance and required a second round of amplification using nested primers. These low abundance species were easily detected using RT-mPCR within the linear range of amplification (Table 3; Figs. 2*C* and 4). To rule out the possibility of genomic contamination by DNA where introns were included, control PCR amplifications were carried out without prior DNAse treatment and RT. These controls produced either no detectable products or PCR products with a different size (Fig. 2*B*).

In general, GLUT3 and HK-I were the predominant glucose transporter and hexokinase, and their abundance did not differ statistically among the three types of neurons sampled for these mRNA species (Table 3). Despite studies suggesting an important role for GLUT2 as a regulator of neuronal glucosensing in β-cells (13,22,34), GLUT2 mRNA was expressed in <30% of glucosensing neurons and its expression did not differ statistically among the neuronal types (Table 3). The mRNA for insulin-responsive GLUT4 was expressed in about half of all neurons, but there were no significant intergroup differences in expression (Table 3). The possibility that neuronal glucosensing might be regulated by insulin's acting on GLUT4, as it is in peripheral tissues (34,35,52), was raised by the coexpression of GLUT4 and INS-R mRNA in 40–75% of all neurons, regardless of their glucosensing properties (Table 3). Because there were no significant intergroup differences in coexpression, it is less likely that insulin-mediated glucose uptake might serve as an alternate mechanism of

TABLE 3
Relative expression of various mRNA species in GE, GI, and NG neurons

	GE	GI	NG
GK	64 (94/146)*	43 (42/98)†	8 (17/211)‡
HK-I	90 (42/47)	91 (30/33)	93 (65/70)
GKRP	10 (2/20)	0 (0/12)	0 (0/10)
GLUT3	83 (38/46)	96 (26/27)	70 (39/56)
GLUT2	30 (14/46)	22 (6/27)	38 (21/56)
GLUT2+GK	22 (6/27)	14 (2/14)	ND
GLUT4	57 (26/46)	63 (17/27)	46 (26/56)
GLUT4+GK	70 (19/27)	64 (9/14)	75 (3/4)
INS-R+GLUT4	75 (12/16)	60 (6/10)	40 (4/10)
SGLT-1	25 (5/20)	8 (1/12)	10 (1/10)
Kir6.2	42 (23/55)*	42 (16/38)*	15 (17/114)†
Kir6.2+GK	38 (12/32)	45 (9/20)	29 (2/7)
Kir6.1	3 (1/33)*	8 (2/24)*	19 (13/68)†
Kir6.1+GK	3 (1/32)	10 (2/20)	14 (1/7)
SUR-1	18 (6/33)	21 (5/24)	21 (14/68)
SUR-2	3 (1/33)	4 (1/24)	3 (2/68)
MCT-1	65 (20/31)	46 (13/28)	50 (38/76)
MCT-1+GK	50 (8/16)	45 (5/11)	67 (4/6)
MCT-2	10 (3/31)	21 (6/28)	12 (9/76)
MCT-2+GK	13 (2/16)	9 (1/11)	0 (0/6)
LDH-A	89 (8/9)	80 (8/10)	73 (24/33)
LDH-B	89 (8/9)	70 (7/10)	82 (27/33)
Lepr-b	25 (9/36)	32 (10/31)	25 (14/57)
Lepr-b+GK	29 (5/17)	12 (2/17)	0 (0/7)
INS-R	68 (21/31)	67 (8/12)	39 (9/23)
GAD	56 (10/18)	36 (5/14)	50 (14/28)

Data are percent of neurons expressing a given mRNA species, with the number of neurons identified over the total number examined given in parentheses. Coexpression (e.g., GLUT2 + GK) of some RNA species is also listed. *, †, ‡Data with dissimilar superscripts within a given set of mRNA species differ from each other by $P < 0.05$ by χ^2 test. Data with no symbols do not differ statistically from each other.

glucosensing. Neither was there a significant difference among the groups for the expression of SGLT-1, although the total number of neurons evaluated was relatively small (Table 3).

In keeping with its postulated role as a major regulator of neuronal glucosensing (8,10,21,27), GK mRNA was expressed in significantly more GE ($P = 0.001$) and GI neurons ($P = 0.001$) than NG neurons. In addition, GK was expressed in more GE than GI neurons ($P = 0.01$) (Table 3). Of those GE and GI neurons expressing GK mRNA, ~75% responded in the expected fashion to alloxan; that is, alloxan decreased $[Ca^{2+}]_i$ oscillations in GE and increased them in GI neurons held at 2.5 mmol/l glucose (Table 4; Fig. 3). However, an appreciable number of glucosensing neurons that expressed GK did not respond to alloxan (Table 4). In the pancreatic β-cell, GLUT2 is the preferred transporter for entry of alloxan into the cell (53); however, only 20–30% of the GE and GI neurons that expressed both GK and GLUT2 also responded to alloxan (Table 4). On the other hand, none of the small number of alloxan-unresponsive glucosensing neurons evaluated expressed both GK and GLUT2 mRNA (Table 4). GLUT2 was also detected in 25–45% of GE and GI neurons without GK mRNA expression, regardless of their alloxan responsiveness (Table 4). In addition, only ~10% of the small number of GE neurons and none of the GI neurons sampled expressed GKRP (Table 4).

The K_{ATP} channel is considered the effector of glucosensing in GE neurons (5,54). However, the Kir6.2 subunit was expressed in only 42% of the GE and GI neurons. Nevertheless, this number significantly exceeded the expression in NG neurons ($P = 0.01$) (Table 3). NG neurons were sixfold more likely to express Kir6.1 than GE ($P = 0.05$) and GI neurons ($P = 0.05$) (Table 3). Surprisingly, the expression of SUR-1 and -2 mRNA was only half that of Kir6.2 or Kir6.1 (Table 3). Because these mRNA species were present in very low abundance and there was a mismatch between the expression of Kir6.1 or Kir6.2 and that of SUR-1 or -2, it is likely that we significantly underestimated their presence by our methods. Because both GI and NG neurons increased their $[Ca^{2+}]_i$ oscillations in the presence of tolbutamide (10), we did not attempt to correlate the presence of these mRNA species with tolbutamide responsiveness, which appears to be a better indicator of the presence of K_{ATP} channel components than our current RT-PCR methods.

Lactate transport into neurons produced by astrocytes has been proposed as an alternate source of energy for neuronal metabolism (8,42). For this to occur, lactate must be transported using MCT (55) followed by conversion to pyruvate using lactate dehydrogenase (LDH) (42,56). Here, MCT-1 was expressed in 46–65% of neurons, regardless of their glucosensing properties (Table 3). About half of all glucosensing neurons and 67% of NG neurons coexpressed both GK and MCT-1. MCT-2 was expressed in <25% of all neurons, with no significant intergroup differences among neuronal types (Table 3). Finally, 70–89% of neurons expressed both isoforms of LDH, regardless of their glucosensing properties (Table 3).

There were no significant intergroup differences in the expression of the leptin receptor b (Lepr-b) splice variant mRNA or the coexpression of Lepr-b and GK (Table 3). On

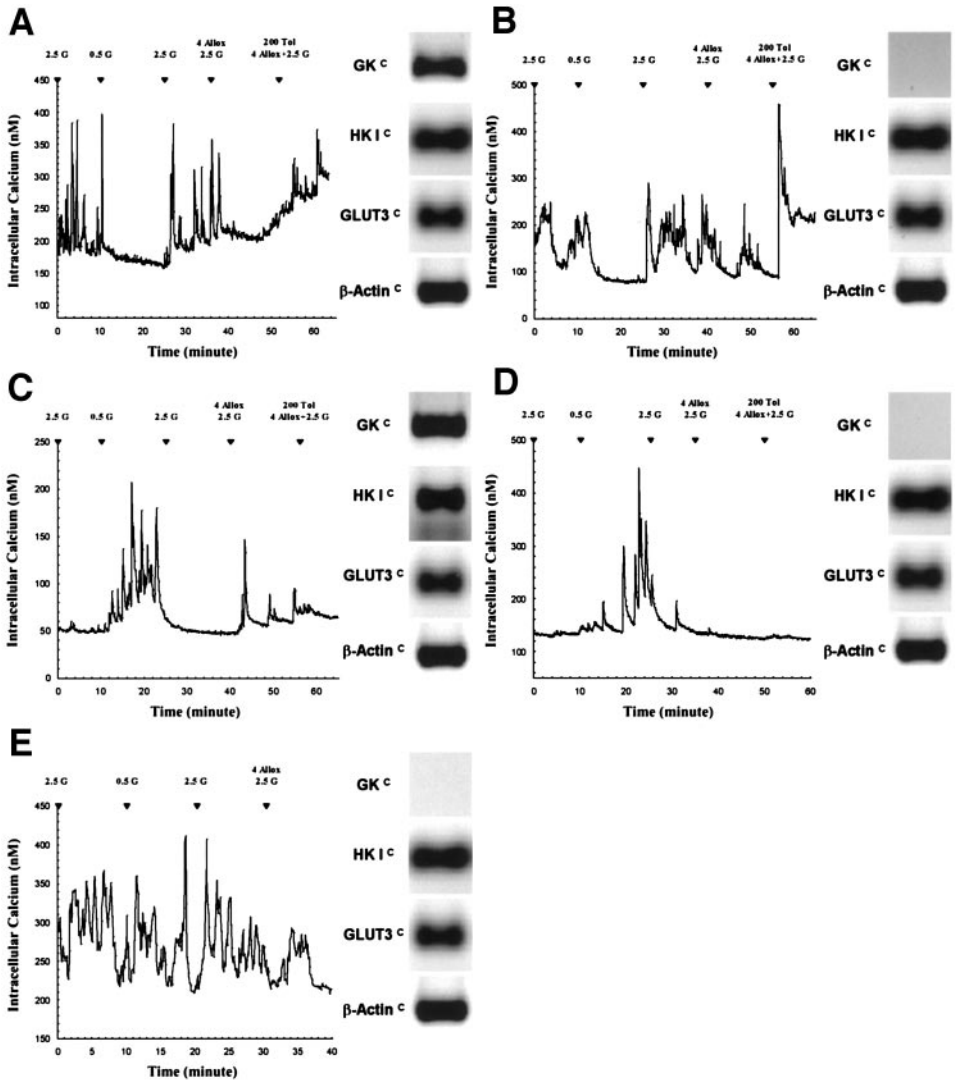

FIG. 3. Alloxan responsiveness in single VMN glucosensing neurons (*right panels*) and the coexpression of GK, HK-I, and GLUT3 mRNA (*left panels*). *A*: Alloxan- and tolbutamide-responsive GE neuron expressing GK, HK-I, and GLUT3. Here $[Ca^{2+}]_i$ oscillations were suppressed by the addition of 4 mmol/l alloxan (Allox) in the presence of 2.5 mmol/l glucose and reinstated by 200 μmol/l tolbutamide (Tol) in the presence of alloxan. *B*: Alloxan-unresponsive, tolbutamide-responsive GE neuron expressing HK-I and GLUT3 but not GK. *C*: Alloxan-responsive, tolbutamide-unresponsive GI neuron expressing GK, HK-I, and GLUT3. Here $[Ca^{2+}]_i$ oscillations were inhibited at 2.5 mmol/l glucose but stimulated by 4 mmol/l alloxan at 2.5 mmol/l glucose. *D*: Alloxan-unresponsive GI neuron expressing HK-I and GLUT3 but not GK. *E*: NG neuron expressing HK-I and GLUT3. Superscript "C" indicates that the amplicon was generated by conventional sc-RT-PCR.

the other hand, 68% of GE and GI neurons expressed INS-R mRNA, a percentage that tended to be higher than that for NG neurons ($P = 0.085$) (Table 3). Of all the VMN neurons assessed, up to one-half were γ-aminobutyric acid (GABA)-ergic, as demonstrated by the expression of GAD mRNA (Table 3). However, there were no intergroup differences in GAD expression.

DISCUSSION

The current studies used glucose-induced changes in $[Ca^{2+}]_i$ oscillations in dissociated VMN neurons to characterize their glucosensing properties and mRNA species by sc-RT-PCR. The influx of extracellular Ca^{2+} occurs after membrane depolarization in β-cells (3). Our data suggest that similar changes in Ca^{2+} flux are also related to changes in neuronal activity in both GE and GI neurons. Glucose-induced changes in dissociated VMN neuron $[Ca^{2+}]_i$ oscillations are qualitatively similar to glucose-induced electrophysiological responses of VMN glucosensing neurons in slice preparations (4,11). One advantage of dissociated neurons is that their activity is not affected by presynaptic inputs as are neurons in slice preparations (4). However, Ca^{2+} signaling is complex and it is not certain that changes in intracellular calcium necessarily completely correlate with changes in action potential. Also, injury to neurons during the dissociation process may lead to spurious classification of glucosensing neurons as NG neurons, particularly in those neurons that showed no spontaneous $[Ca^{2+}]_i$ oscillations. This might account for the finding of GK mRNA in apparent NG neurons. However, the similarities in behavior of dissociated neurons studied using $[Ca^{2+}]_i$ imaging to neurons studied electrophysiologically in slice preparations suggest that the evoked $[Ca^{2+}]_i$ oscillations are a reasonable surrogate for classifying neurons with regard to their glucosensing properties.

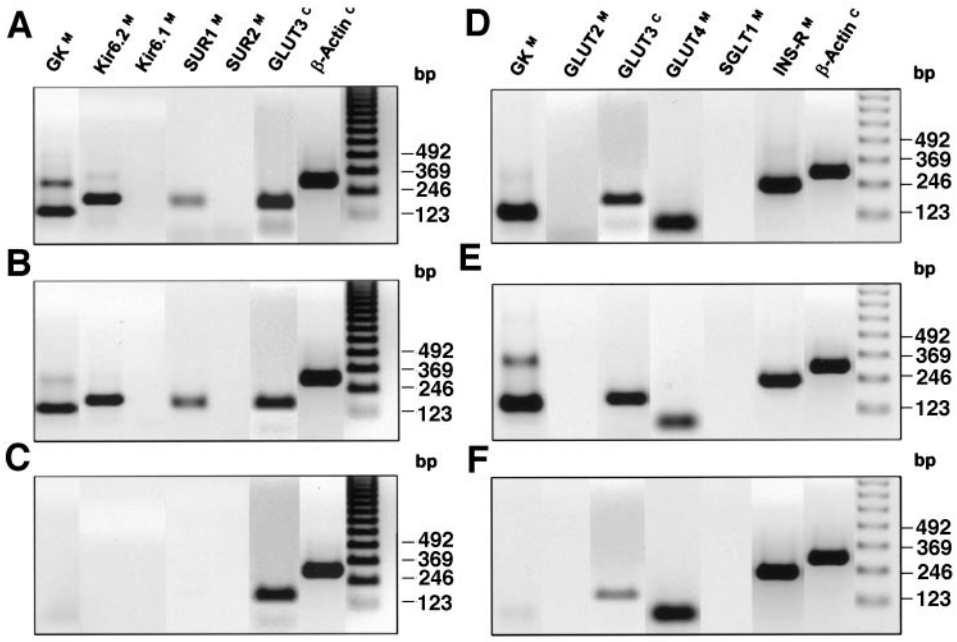

FIG. 4. Coexpression of various mRNA species in single GE (*A* and *D*), GI (*B* and *E*), and NG VMN neurons (*C* and *F*). *Left*: Coexpression of GK and K_{ATP} channel subunits. *Right*: Coexpression of GK, various glucose transporters, and INS-R. Templates for *left* and *right panels* are from the different neurons. Fragment sizes are in agreement with those predicted from the mRNA sequences (Table 1). Superscript "C" and "M" indicate that the amplicons were generated by conventional PCR and mPCR, respectively.

Here we correlated the physiological and pharmacological responses of dissociated VMN glucosensing neurons with their expression of various mRNA species purported to function in pancreatic and neuronal glucosensing. Although the presence of a specific mRNA species does not guarantee the expression of a functional protein product, it is likely that such translational events do occur in most cases. Also, because of limitations in detection thresholds and sampling bias, the quoted percentages for given mRNA species must be taken as estimates as it is likely that some species were underestimated (e.g., Kir6.1 and Kir6.2, SUR-1 and -2). However, although the expression of GK was quite low, GK mRNA was always detectable within the linear range of sensitivity of a single round of amplification. It is still possible that some neurons express GK mRNA at very low levels, but have sufficient enzyme protein to use GK for glucosensing. However, our previous study (10) suggested that, as in the current estimate of GK mRNA expression, up to 30% of GE neurons did not respond to a panel of four different GK inhibitors. We also used the GK inhibitor alloxan to correlate a functional measure of GK activity with the expression of GK mRNA. On the other hand, it is unlikely that there were a significant number of false-positive results because scrupulous attention was paid to eliminating genomic DNA and to ensuring that the number of amplification cycles used for a given species was well within the linear range of amplification for each species. Finally, there was a potential sampling bias for the way in which neurons were chosen here as the percentages quoted were dependent on the fact that sample neurons were the ones that survived the dissociation procedure and incubation period before testing.

Given these caveats, there are many similarities, but also some differences, between GE neurons and β-cells. GK and Kir6.2 mRNA expression were less common in GE neurons than expected from previous studies (14,57) and studies in the β-cell (58–60), although the Kir6.2 results must be interpreted with caution. In all, ~60% of GE neurons expressed GK, the proposed regulator of β-cell glucosensing (20,61,62). Also, <50% of GE neurons expressed Kir6.2 or Kir6.1 and even fewer expressed SUR-1 or -2 mRNA. However, given the pharmacological responses of GE neurons to alloxan and other GK inhibitors (8,10), it is still likely that GK and the K_{ATP} channel are the most common mediators of glucosensing in GE neurons.

TABLE 4
Comparison of potential regulators of VMN neuronal glucosensing with regard to responsiveness or nonresponsiveness to 4 mmol/l alloxan and expression of potential regulators of glucosensing

	GK	GK(+) GLUT2(+)	GK(−) GLUT2(+)	GKRP	HK-1
GE					
Alloxan(+)	76 (28/37)	30 (6/20)	38 (3/8)	9 (1/11)	87 (33/38)
Alloxan(−)	45 (9/20)	0 (0/7)	45 (5/11)	11 (1/9)	100 (9/9)
GI					
Alloxan(+)	74 (14/19)	17 (2/12)	40 (2/5)	0 (0/5)	92 (11/12)
Alloxan(−)	18 (2/11)	0 (0/2)	25 (2/8)	0 (0/7)	90 (19/21)

Data are percent, with number of neurons identified over the total number examined given in parentheses.

On the other hand, GI neurons are similar to pancreatic α-cells in that both express GK (10,23). Although the K_{ATP} channel is unlikely to mediate glucosensing in GI neurons, the expression of GK mRNA in 43% of GI neurons and their responses to various GK inhibitors (10) make it likely that GK plays a regulatory role in glucosensing in some GI neurons. Finally, it is still unclear how GK, with its high K_m, might mediate neuronal glucosensing at physiological brain glucose levels (4,10,11). Like the β-cell, which expresses a similar isoform of GK but not GKRP (27,32), glucosensing neuron GKRP expression was too infrequent for it to be a significant regulator of GK activity that might bring the functional K_m of GK activity down to appropriate brain glucose levels (11,12,63).

There are also several differences between GE neurons and pancreatic β-cells. First, virtually all VMN neurons expressed GLUT3, as well as the low-K_m HK-I. Rat β-cells express predominantly GLUT2 and GK (13), whereas only a small percent of VMN neurons expressed GLUT2 in our study. In fact, the neuronal expression of GLUT2 was surprising given prior reports suggesting that this is predominantly an astrocytic transporter (34). On the other hand, a significant number of neurons coexpressed GLUT4 and INS-R, although there were no significant intergroup differences by glucosensing property. This suggests that a significant proportion of neurons might use insulin-mediated glucose uptake to fuel their metabolic needs, but that this is less likely to be a primary mechanism for glucosensing. A smaller but significant number of GE neurons also expressed SGLT-1 mRNA, in keeping with the finding that phloridzin, a SGLT-1 inhibitor, reduces firing of GE neurons (8). In addition, unlike β-cells (64–67), both GE and GI neurons expressed both MCT-1 and LDH-A mRNA. This should enable those neurons to use lactate and ketone bodies as alternate energy substrates and act as regulators of glucosensing (8). Finally, whereas most β-cells are GABAergic (68), only half of the VMN GE neurons assessed expressed the mRNA for GAD, the GABA synthesizing enzyme (69). Thus, although many GE neurons may sense glucose similarly to β-cells, there are likely to be other populations of VMN GE and GI neurons that sense glucose through alternate pathways.

Hypothalamic neurons are also involved in sensing and regulating peripheral signals related to energy homeostasis other than glucose (70). These include metabolic signals such as lactate and ketone bodies and hormones related to adipose mass such as insulin and leptin. The presence of MCT, LDH, INS-R, and Lepr-b mRNAs suggests that glucosensing neurons can serve a more generalized role as metabolic sensors. Both insulin and leptin circulate in proportion to carcass adiposity (70–72) and regulation of insulin secretion is critical for the control of glucose metabolism. At high glucose concentrations, both leptin and insulin can either inhibit (73–75) or excite (76) hypothalamic glucosensing neurons. Although inhibition appears to be mediated by an effect on the K_{ATP} channel (73), it is unclear how leptin- and insulin-mediated neuronal excitation occurs. Here, ~33% of glucosensing neurons expressed Lepr-b and ~70% expressed INS-R mRNA. Other metabolic substrates besides glucose might also regulate firing in glucosensing neurons. The current studies suggest that glucosensing neurons could potentially use astrocyte-derived lactate (8,39) as an alternate regulator of firing rate. In addition, ketone bodies produced in the periphery during starvation (77), untreated diabetes (78), and the neonatal period (40) might serve as regulators of neuronal firing. Both lactate and ketone bodies gain access to the brain via MCTs (43). Here, about half of all VMN neurons, regardless of their glucosensing properties, expressed MCT-1, whereas a much smaller number expressed MCT-2. This finding is contrary to prior reports in adult and neonatal rat and mouse brains (44,79,80). Our current findings for MCTs might be influenced by our use of 14- to 21-day-old pups, as the expression of MCT-1 mRNA is highest at postnatal day 15 (81). Results for other mRNAs might also reflect the developmental stage of these young animals. Regardless of the MCT subtype, both LDH-A and -B were ubiquitously expressed in all neuronal types. Thus, both glucosensing neurons might convert exogenous lactate to pyruvate using MCT-1 and LDH-B (53) to alter their firing rate (8). This is different from the β-cell, which cannot use lactate to increase insulin secretion (61,82,83). Thus, glucosensing neurons have the potential to use both peripheral hormones and metabolites to regulate their activity.

In summary, a population of VMN neurons possesses the cellular machinery to enable them to use glucose as a signaling molecule. A substantial proportion of these neurons appear to use GK as a gatekeeper for glucose-induced excitation or inhibition of activity. However, it is clear that there must be other, non–GK- and non–K_{ATP}-dependent pathways in some VMN glucosensing neurons. In addition, many VMN glucosensing neurons also have the molecular pathways required for responding to both hormonal and other metabolic signals involved in the regulation of energy homeostasis.

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