

# Effects of glucose and insulin on glucokinase activity in rat hypothalamus

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## Abstract

In an attempt to study the role of glucokinase (GK) and the effects of glucose and peptides on *GK* gene expression and on the activity of this enzyme in the hypothalamus, we used two kinds of biological models: hypothalamic GT1-7 cells and rat hypothalamic slices. The expression of the *GK* gene in GT1-7 cells was reduced by insulin (INS) and was not modified by different glucose concentrations, while GK enzyme activities were significantly reduced by the different peptides. Interestingly, a distinctive pattern of GK activities between the ventromedial hypothalamus (VMH) and lateral hypothalamus (LH) were found, with higher enzyme activities in the VMH as the glucose concentrations rose, while LH enzyme activities decreased at 2.8 and 20 mM glucose, the latter effect being prevented by incubation with INS. These effects were

produced only by D-glucose and the modifications found were due to GK, but not to other hexokinases. In addition, GK activities in the VMH and the LH were reduced by glucagon-like peptide 1, leptin, orexin B, INS, and neuropeptide Y (NPY), but this effect was only statistically significant for NPY in LH. Our results indicate that the effects of both glucose and peptides occur on GK enzyme activities rather than on *GK* gene transcription. Moreover, the effects of glucose and INS on GK activity suggest that in the brain GK behaves in a manner opposite to that in the liver, which might facilitate its role in glucose sensing. Finally, hypothalamic slices seem to offer a good physiological model to discriminate the effects between different areas.

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## Introduction

Glucokinase (GK) is a type IV isoenzyme that belongs to the family of hexokinases (ATP: D-hexose 6-phosphotransferase; EC 2.7.1.1) and catalyzes the formation of glucose 6 phosphate in eukaryotic cells. This isoenzyme (Dipietro *et al.* 1962, Viñuela *et al.* 1963) has a molecular mass of 52 kDa and a low affinity for glucose and is not subject to feedback inhibition by glucose 6-phosphate.

GK activity is expressed in liver (Printz *et al.* 1993), the pancreatic islets of Langerhans (Matschinsky & Ellerman 1968), jejunal enterocytes, neuroendocrine cells, and brain (Jetton *et al.* 1994, Navarro *et al.* 1996, Roncero *et al.* 2000). *GK* is encoded by a single gene, but the presence of alternative promoters (Magnuson & Shelton 1989) allows the cell-specific expression of this protein with differential regulation. Thus, liver GK is regulated by insulin (INS) (Printz *et al.* 1993), whereas the pancreatic enzyme seems to be controlled posttranslationally by glucose levels (Matschinsky & Ellerman 1968). Along the same lines, brain GK should be controlled by glucose levels and this is one of the issues studied here.

GK plays a key role in the glucose metabolism of the liver and it is considered to be a glucose sensor in pancreatic  $\beta$ -cells involved in glucose-dependent INS release (Matschinsky 1990), as well as in hepatocytes and

hypothalamic neurons (Alvarez *et al.* 1996, Navarro *et al.* 1996, Kang *et al.* 2006). At least two kinds of glucose sensor neurons have been identified in brain. Glucose-excited (GE) neurons are present mainly in the ventromedial hypothalamus (VMH) neurons of the hypothalamus and they are excited by increased glucose levels in the extracellular space, with alterations in their firing rates (Oomura *et al.* 1969). By contrast, the glucose-inhibited (GI) neurons, mainly present in the lateral hypothalamus (LH) area, are excited by the decrease in glucose in the extracellular space. GK seems to be the mediator of glucose sensing in both GE and GI neurons (Dunn-Meynell *et al.* 2002). When GE neurons are exposed to high-glucose levels, GK activation produces an increase in the ATP/ADP ratio, after which the  $K_{ATP}^+$  channel (Dunn-Meynell *et al.* 1998) is inactivated, producing membrane depolarization. As a consequence, a  $Ca^{2+}$  influx through a voltage-activated  $Ca^{2+}$  channel occurs. Although  $K_{ATP}^+$  channel distribution is ubiquitous in brain, only selective areas of this organ show glucose-sensing properties, which seems to be governed by the activity of GK. Contrariwise, when glucose levels decrease,  $Ca^{2+}$  oscillations increase in GI cells but are reduced in GE neurons (Niimi *et al.* 1995).

It has been suggested that GK may recognize elevations in plasma glucose levels after food ingestion and its interactions

with the glucokinase regulatory protein (GKRP) may facilitate the functioning of glucose-sensing sites located in selective neurons of the hypothalamus and hindbrain (Alvarez *et al.* 1996, 2002). In keeping with this proposal, groups of these cells containing orexigenic and anorexigenic peptides and their receptors may produce integrated responses to modifications in metabolites, enabling them to act as sensors of glucose circulating levels. To test this working hypothesis, we determined the effects of different glucose concentrations and orexigenic and anorexigenic peptides on *GK* gene expression and *GK* activity in GT1-7 and rat hypothalamic slices.

## Materials and Methods

### Experimental animals

All procedures involving animals were approved by the appropriate institutional review committee and met the guidelines for the care of animals specified by the European Community. Male Wistar rats weighing 150–175 g were fed *ad libitum* with a standard pellet diet and housed at a constant temperature (21 °C) on a 12 h light:12 h darkness cycle with lights on at 0800 h. Rats were killed by decapitation under non-fasting state, and the whole brain was rapidly removed and placed in ice-cold medium. All procedures were carried out according to the European Union ethical regulations for animal research.

### Cell cultures and *GK* promoter activity assays

The GT1-7 cell line (generously provided by Prof. P Mellon, Department of Reproductive Medicine, School of Medicine, University of California, San Diego, CA, USA), transformed from mouse hypothalamic neurosecretory cells (Mellon *et al.* 1990), is an excellent tool for neuronal studies (Wetsel 1995). GT1-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) containing 4.5 g/l glucose, 10% fetal bovine serum (Biomedica, Bousens, France), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine (Biomedica). The wild-type reporter gene construct (generously provided by Dr Magnuson, Department of Molecular Physiology and Biophysics, Vanderbilt University Medical Center, Nashville, TN, USA) was created by cloning 1014 bp pancreatic rat *GK* promoter (−1000 to +14) into the promoterless luciferase vector pSVOAPL2L (Shelton *et al.* 1992). Transfection was performed using calcium phosphate precipitates containing 3 µg luciferase expression vector with the *GcK* promoter, and 1 µg internal control plasmid pCMV-βgalactosidase for each well of a 12 multiwell plate. GT1-7 cells were incubated with calcium phosphate precipitates for 14–16 h and then rinsed and kept in fresh medium for 24 h. Following this, the cells were incubated overnight in the presence of medium containing 2% fetal bovine serum (FBS) and 5.5 mM glucose. The

medium was then removed and replaced by DMEM containing 2% Fetal bovine serum (FBS) and different glucose and/or peptide concentrations. After 5 h of treatment, the cells were lysed, and luciferase activity was determined with a luminometer (BG-P luminometer, GEM Biomedical, Hamden, CT, USA). Luciferase activity levels, normalized with β-galactosidase activities are expressed as fold stimulation of control (5.5 mM glucose) values.

### RNA isolation and semiquantitative RT-PCR analysis of *GK* mRNA

Total RNA from culture were extracted with TRIZOL (Life Technologies). The purity of the RNA was assayed by electrophoresis in agarose gel. One microgram total RNA was transcribed to obtain 20 µl cDNA (RETROscript Ambion, Austin, TX, USA). Five microlitres cDNA were used as template for the PCR, following the manufacturer's instructions (Biotools B&M Labs SA, Madrid, Spain). The pancreatic-specific primers for the *GK* amplification were 5'AATCTTGC GGAACTGAG3' and 5'CACGTAGGTGGTAACATCTTTACAC3'. To control the differences in initial RNA levels and tube-to-tube variations in PCR, a primer pair for 18S was included in each PCR amplification. The control 18S amplification primers were 5'TCAAGAACGAAAGTCCGGAGG 3' and 5'GGACATCTAAGGGCATCACA3'. The conditions used were as follows: 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, and a final extension cycle at 72 °C for 10 min.

### Procedure for hypothalamic-slice explants culture

Hypothalamic-slice explants cultures were performed as described previously (House *et al.* 1998, Arima *et al.* 2001). Male Wistar rats were killed by decapitation and the brains were quickly removed and immersed in cold (4 °C) MEM medium containing 25 mM HEPES, 20% heat-inactivated horse serum, 4 mM glutamine, 6.5 mg/ml glucose, and 100 U/ml penicillin–streptomycin. Following this, the hypothalamus was removed from the brain and sectioned at 300 µm thickness on a McIlwain tissue chopper (Mickle Laboratory Engineering Co., Surrey, UK). Coronal slices were separated and placed in MEM supplemented with 25 mM HEPES and Hank's salt (Life Technologies) enriched with the above components. In an attempt to stabilize the cultures, hypothalamic slices were cultured at 37 °C in an atmosphere of air and CO<sub>2</sub> (95:5% respectively) in the medium (MEM, 25 mM HEPES with Hank's salt, 20% heat-inactivated horse serum, 4 mM glutamine, 6.5 mg/ml glucose, and 100 U/ml penicillin–streptomycin) for 5 h. Then, the hypothalamic slices were transferred to a medium containing 2% FBS and 5.5 mM glucose and incubated for 16 h. The cell viability of the hypothalamic slices was determined by the addition of 0.5 µg/ml propidium iodide to the culture medium or by incubating the slices in a solution

containing 1 ml Trypan blue solution (0.8 mM in PBS) and 1 ml culture medium. After at least 15 min of incubation, propidium staining was assessed with an inverted fluorescence microscope, using a standard rhodamine filter set. In the case of trypan blue, cell viability was determined under a light microscope 5 min after incubation with the stain.

To examine the effects of different glucose concentrations and/or peptides on GK enzyme activities in hypothalamic slices, these were incubated with 0.05, 0.5, 2.8, 5.5, 10, or 20 mM glucose and/or peptides at the indicated concentrations for 3 h. At the end of the incubations, special care was taken to identify and isolate by micropunching the VMH and LH areas according to the stereotaxic coordinates (Paxinos & Watson 1986).

#### Western blot assay

For immunoblot detection of GK, 80 µg total protein from VMH and LH areas were resolved by electrophoresis through a SDS-PAGE (9%). After blocking for 1 h in PBS-0.1% Tween20 containing 5% BSA, the membranes were incubated with a rabbit anti-GK antibody (1/2000; a generous gift from Dr J Guinovart, Department de Bioquímica i Biologia Molecular, Universitat de Barcelona, Barcelona, Spain). After washing off excess antibody, the membranes were incubated with an anti-rabbit IgG biotinylated (1:5000) for 1 h and finally with Streptavidin (Amersham; 1:5000) conjugated to horseradish peroxidase. Chemiluminescence detection was carried out in the presence of Chemiluminescent HRP Substrate (Millipore, MA, USA). Specificity of GK antibody was tested by the preincubation with or without the antigenic peptide (KLHPSFKERFHASVR).

To map the functional activity of VMH and LH, the expression of the immediate early gene *c-fos* was determined (Sharp *et al.* 1993, Chaudhuri *et al.* 2000). Thus, c-Fos protein was separated by 9% SDS-PAGE and determined by immunodetection in VMH and LH homogenates from hypothalamic slices incubated for 3 h in the presence of low and high-glucose concentrations (1 and 20 mM).

#### Assay of glucose-phosphorylating activities

The GT1-7 cells or VMH and LH hypothalamic areas were homogenized in ice-cold lysis medium (50 mM HEPES, 150 mM KCl, 5 mM MgCl<sub>2</sub>, and 1 mM EDTA, pH 7.4) supplemented with 1 mM dithiothreitol, 1 mM phenylmethyl-sulfonyl fluoride, and 10 µM leupeptin. Homogenates were used to measure enzyme activities.

Glucose-phosphorylation activity analysis involved assays at two glucose concentrations: 0.5 mM for low *K<sub>m</sub>* hexokinase activities, a concentration at which GK is essentially inactive (Bedoya *et al.* 1984, Nishio *et al.* 2006), and 25 mM glucose, a concentration at which all phosphotransferase activities were measured. Glucose phosphorylation rates were measured using an isotopic method (Bedoya *et al.* 1984). In brief, the

reaction was initiated by the addition of 10 µl soluble fractions of GT1-7 cells or hypothalamic areas to 110 µl total volume of the reaction mixture containing 50 mM HEPES (pH 7.4), 150 mM KCl, 5 mM MgCl<sub>2</sub>, 5 mM Mg-ATP, 10 mM NaF, and 0.5 or 25 mM D-[U-<sup>14</sup>C]glucose (Amersham Life Science, purity ≥ 98.9%), with specific activities of 3.7 or 0.7 mCi/mmol respectively. The reaction was continued at 30 °C for 20 min and stopped by the addition of 30 µl 2 M glucose and 0.25 mM EDTA. Duplicate 60 µl samples were spotted onto diethyl amino ethyl (DEAE)-cellulose filters. After washing with distilled water, the filters were dried, and the radioactivity, i.e. the formation of [<sup>14</sup>C]glucose phosphoric esters, was counted by liquid scintillation spectrometry. Blanks without ATP or homogenate were included in each experiment. GK activity was calculated by subtracting the glucose-phosphorylating activity at 0.5 mM glucose from the total activity measured at substrate concentrations of > 5 mM.

#### Statistical analysis

All values are presented as mean ± S.E.M. Comparisons among groups were made using ANOVA. *P* ≤ 0.05 was considered statistically significant.

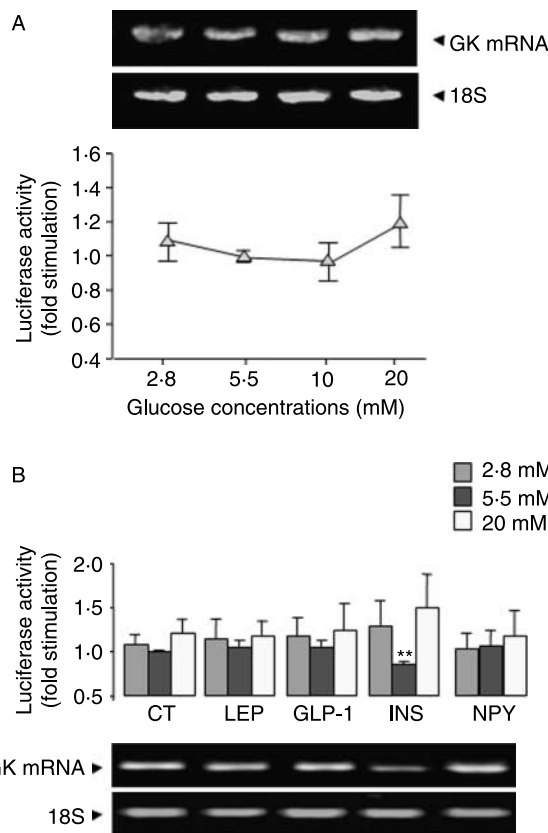
## Results

#### Effects of glucose and different peptides on GK gene expression in GT1-7 cells

The action of different glucose concentrations and peptides on GK gene promoter expression was tested in transiently transfected GT1-7 cells. Neither different glucose concentrations (2.8, 5.5, 10, or 20 mM) nor leptin (LEP), glucagon-like peptide 1 (GLP-1) or neuropeptide Y (NPY) were able to modify the promoter activity (Fig. 1) after 5 h of incubation. At the same time, we did not obtain variations in the endogenous GK mRNA levels. However, INS was able to significantly decrease both promoter activity and endogenous GK mRNA.

#### Effects of glucose and different peptides on GK activities in GT1-7 cells

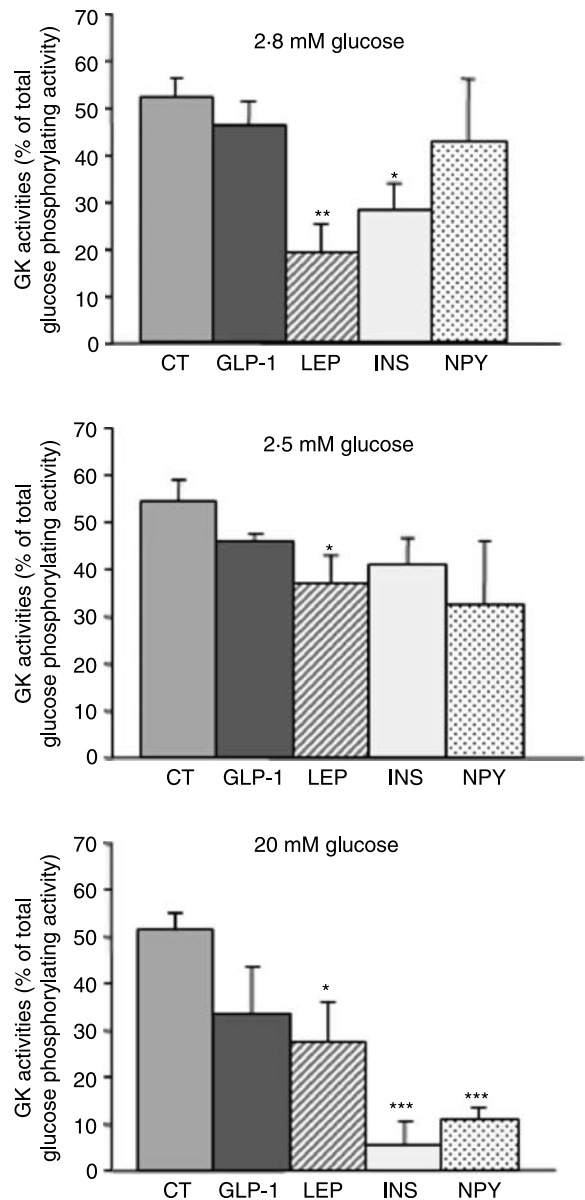
Although glucose and different peptides were unable to modify GK gene expression, in an attempt to gain better physiological insight into this process, we tested the activities of GK after GT1-7 cells were incubated with several glucose concentrations and in the absence or presence of GLP-1, LEP, and INS. Whereas, the different glucose concentrations did not change enzyme activities, all peptides tested, except GLP-1 decreased GK activities, although this was modulated by the concentrations of glucose. Thus, LEP decreased GK activity at all the glucose concentrations tested, while INS did so at 2.8 and 20 mM, and NPY only at 20 mM. (Fig. 2).



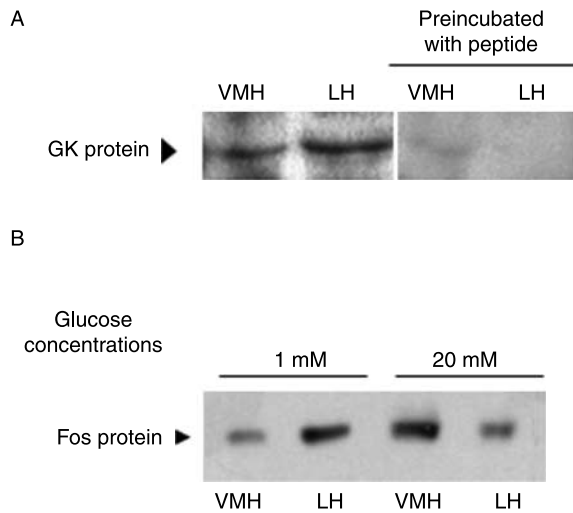
**Figure 1** Effects of glucose and orexigenic or anorexigenic peptides on GK promoter activity and endogenous GK mRNA expression in GT1-7 cells. (A) and (B) show the fragments of DNA obtained after PCR amplification of cDNA, and graphics represent the data obtained from the promoter activity studies. Wild-type (panels) GT1-7 cells or cells transiently transfected with a luciferase reporter plasmid containing *Gck* promoter (graphics), were incubated with 2.8, 5.5, 10, or 20 mM glucose (A) and different peptides (B) 10 nM leptin (LEP), 10 nM GLP-1, 10 nM insulin (INS), or 100 nM neuropeptide Y (NPY). Data in the graphics are represented as fold increase of GK-luciferase activity (ratio of luciferase to  $\beta$ -galactosidase activity) relative to the data obtained to 5.5 mM glucose treatment  $\pm$  s.e.m. The experiment was repeated at least seven times in duplicate. \*\* $P \leq 0.01$  (INS treatment at 5.5 mM glucose versus 5.5 mM glucose without peptide).

#### Studies in hypothalamic-slices explants

A further step was to study the effect of glucose on rat hypothalamic slice explants, which constitute a good physiological model because they preserve some tissue architecture and the functional connections involved in glucose sensing and feeding behavior. In these hypothalamic slices cell viability was maintained, as determined by the exclusion of propidium iodide and trypan blue. In addition, after western blot analyses for GK was performed, a specific band corresponding to GK protein was found in both VMH and LH areas. The signal was specific since it disappeared, when the membrane was preincubated with the antigenic peptide (Fig. 3A). VMH and LH areas kept their functional



**Figure 2** Effects of glucose and orexigenic or anorexigenic peptides on GK activities in GT1-7 cells. GT1-7 cells were incubated with 2.8, 5.5, and 20 mM glucose in the absence (CT) or presence of different peptides: 10 nM GLP-1, 10 nM leptin (LEP), 10 nM insulin (INS), and 100 nM neuropeptide Y (NPY). Glucose phosphorylating activities were determined in soluble fractions from GT1-7 cells. Total glucose phosphorylating activity was determined at 25 mM glucose, while low-Km hexokinase (HK) activities were determined at 0.5 mM glucose, and GK activity was obtained by the difference of individual values at 25 and 0.5 mM glucose. Values of GK activities are given as means  $\pm$  s.e.m. and correspond to the percentage of total phosphorylating activities for at least four independent experiments repeated in duplicate. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , and \*\*\* $P \leq 0.001$  (incubated with peptides versus CT).

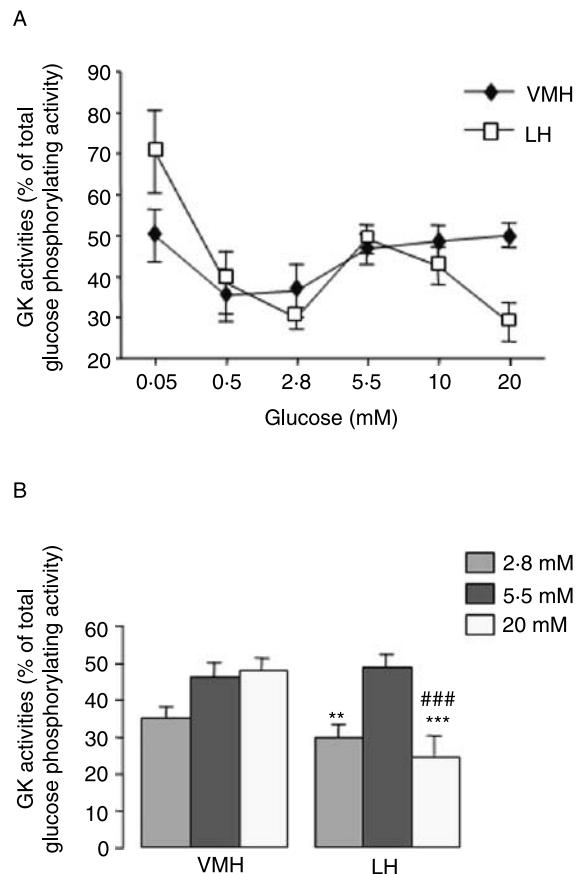


**Figure 3** GK protein expression and effects of glucose on Fos protein levels in VMH and LH areas. (A) VMH and LH areas were dissected from rat hypothalamus, homogenized, and the proteins separated by SDS/PAGE. GK immunoreactivity was detected with an anti-GK antibody (left), while no bands were found when the membrane was preincubated in the presence of GK-antibody plus the immunogenic peptide (right). The figure shows a representative western blot. (B) Hypothalamic slices were incubated for 3 h in a medium that contained either 1 mM or 20 mM glucose. Thereafter, VMH and LH areas were dissected from the hypothalamic slices and homogenized. After SDS-PAGE of hypothalamic extracts, Fos protein was detected by incubation with an anti-Fos antibody. The figure shows a representative western blot.

activity since a high-glucose concentration (20 mM) produced a significant increase in the amount of Fos protein in VMH, but reduced it in LH (Fig. 3B). By contrast, 1 mM glucose had the contrary effect, with a greater appearance of Fos protein band in LH, whereas it was significantly reduced in VMH.

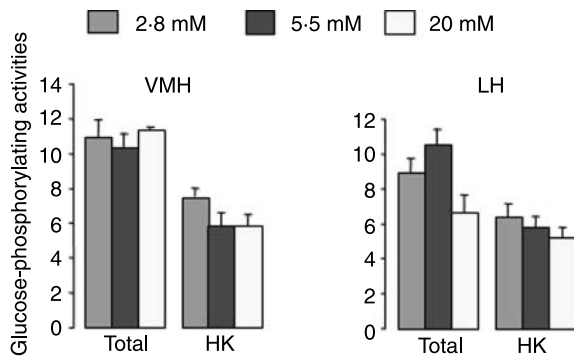
#### Effect of glucose on GK activities in hypothalamic-slice explants

Glucose concentrations ranging from 0.05 to 20 mM produced changes in GK activities (Fig. 4), depending on the concentration in the extracellular space and on the hypothalamic area tested. Thus, in the VMH a tendency for GK activities to increase was observed when the glucose concentrations rose, while in the LH the tendency was for GK activities to decrease at higher glucose levels (Fig. 4A). However, in the LH a peak of GK activity also appeared at 5.5 mM glucose as compared with 2.8 and 20 mM glucose, GK activities being significantly lower in LH than in VMH at 20 mM glucose (Fig. 4B). The glucose-induced changes in total glucose-phosphorylating activities in the VMH and the LH must be due to the modifications in GK activities because no changes in low-Km hexokinases (HKs) were observed, as shown in Fig. 5, where total glucose phosphorylating and HKs are plotted as a function of the glucose concentration present in the extracellular space. These effects of glucose on



**Figure 4** Effects of glucose concentrations on GK enzyme activities in hypothalamic slice explants. The hypothalamic slice-explant cultures were incubated for 3 h in a medium that contained 0.05 mM, 0.5 mM, 2.8, 5.5, 10, or 20 mM glucose. VMH and LH nuclei were dissected from the hypothalamic slices after the incubation period and glucose phosphorylating activities were determined in soluble fractions from these areas, where total glucose phosphorylating activity was determined at 25 mM glucose, while low-Km hexokinase (HK) activities were determined at 0.5 mM glucose, and GK activity was obtained by the difference of individual values at 25 mM and 0.5 mM glucose. Values of GK activities are given as means  $\pm$  s.e.m. and correspond to the percentage of total phosphorylating activities for at least four independent experiments repeated in duplicate. \*\* $P \leq 0.01$  (2.8 mM glucose versus 5.5 mM), \*\*\* $P \leq 0.001$  (20 mM glucose versus 5.5 mM) and \*\*\* $P < 0.001$  (LH area versus VMH).

GK activity seem to be specific, because when we used the L-glucose form, in which biological activity is lacking, we were unable to detect the effects induced by D-glucose (Table 1). To test whether the actions of high-glucose concentrations on GK activity might be produced by hypertonic changes in the cells, we incubated the hypothalamic slices with different concentrations of either sucrose or glucose. Under these conditions, 20 mM sucrose had no effect as compared with the significant reduction in GK activity induced by 20 mM glucose (Fig. 6).



**Figure 5** Effects of glucose concentrations on glucose phosphorylating activities in VMH and LH areas. The hypothalamic slice-explant cultures were incubated for 3 h in a medium that contained 2.8 mM, 5.5 mM, or 20 mM glucose. VMH and LH nuclei were dissected from the hypothalamic slices after the incubation period and glucose phosphorylating activities were determined in soluble fractions from these areas, where total glucose phosphorylating activity was determined at 25 mM glucose, while low-Km hexokinase (HK) activities were determined at 0.5 mM glucose. Values of glucose phosphorylating activities are given as means  $\pm$  s.e.m. expressed as pmol of [<sup>14</sup>C]glucose 6-phosphate/min per µg protein for at least four independent experiments repeated in duplicate.

#### Effect of INS and other peptides on GK activities in hypothalamic-slice explants

When we tested the effects of different orexigenic and anorexigenic peptides on the GK activities of the VMH and LH (Table 2), we found that GLP-1 and INS did not elicit any changes, whereas LEP, orexin B, and NPY decreased the activities of this enzyme; this effect was statistically significant only in the case of NPY in the LH area.

Although INS did not modify GK activities at 5.5 mM glucose in hypothalamic slices, the effect of higher glucose concentrations (20 mM) reduced GK activity in the LH but not in the VMH and this effect was reversed when tissue slices were incubated with INS (Fig. 7). Thus, when hypothalamic slices were incubated with 5.5 mM glucose and INS no changes were detected in the VMH, whereas in the LH the addition of 20 mM glucose significantly reduced GK activities, which were recovered after addition of the hormone (Fig. 7).

**Table 1** Glucose phosphorylating activities in ventromedial hypothalamus (VMH) and lateral hypothalamus (LH) areas incubated with 20 mM D-glucose or L-glucose. Values represent means  $\pm$  s.e.m. (n) expressed as pmol of [<sup>14</sup>C]glucose 6-phosphate/min per µg protein

	20 mM D-glucose			20 mM L-glucose		
	Total	HK	GK	Total	HK	GK
VMH	11.5 $\pm$ 0.8 (16)	5.8 $\pm$ 0.4 (16)	5.7 $\pm$ 0.6 (16)	12.0 $\pm$ 0.8 (5)	8.0 $\pm$ 0.5 (5)	3.9 $\pm$ 0.6 (5)
LH	7.6 $\pm$ 1.0 (12)	5.6 $\pm$ 0.6 (10)	2.3 $\pm$ 0.6 (12)*	11.3 $\pm$ 1.6 (5)	6.9 $\pm$ 1.1 (5)	4.3 $\pm$ 1.3 (5)

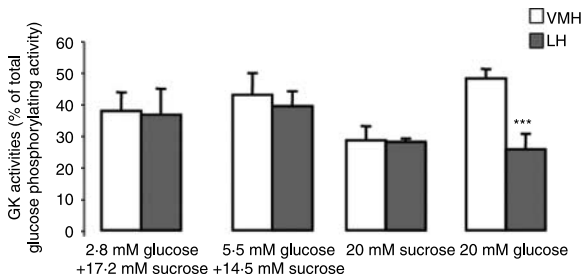
\* $P < 0.001$  when values obtained in VMH and LH incubated at 20 mM D-glucose were compared.

## Discussion

GK has been characterized in the brains of human (Alvarez *et al.* 2005) and experimental animals (Jetton *et al.* 1994, Navarro *et al.* 1996, Roncero *et al.* 2000); the isoform present in the central nervous system being similar to that found in pancreatic  $\beta$ -cells (Roncero *et al.* 2000), where it seems to contribute as a glucose sensor involved in the control of food intake (Alvarez *et al.* 1996). These findings open the door to study the effects of glucose and anorexigenic and orexigenic peptides as substances that may potentially modulate the expression of the GK gene and/or the activity of this enzyme in brain.

To accomplish these aims, we used cells of hypothalamic origin such as GT1-7 to study the transcriptional regulation of the GK gene in cells transiently transfected by vectors containing the promoter fused to the luciferase gene. In addition, we determined the catalytic activities of this enzyme. There is experimental evidence that GT1-7 cells are glucose-sensing cells (Mellon *et al.* 1990, Lee *et al.* 2005) able to respond to glucose deprivation or high-glucose levels, as well as having intrinsic GK activity (data not shown). They represent a model for studying the transcriptional regulation of GK gene since the transfection method cannot be applied to other systems, including hypothalamic slices. However, immortalized cell lines are limited in studies of transcriptional activity because they are not located within a complex neuronal network. Thus, a further step was the use of rat hypothalamic slices, which represent an experimental design of physiological relevance because they preserve some of the tissue architecture and the functional connections involved in feeding behavior. In addition, they contain GE and GI neurons with GK activities, and receptors to different peptides, and they retained a selective c-Fos expression in response to different glucose concentrations, indicating that these hypothalamic areas preserve the functional activity found in the whole animal (Solomon *et al.* 2006). These findings and the distinctive effects of different glucose concentrations and peptides on GK activities in the VMH and the LH underscore the relevance of this experimental design, as the time that remark the selective functional activities of both hypothalamic areas.

GK is considered to be a true glucose sensor present in GE and GI neurons where it seems to be implicated in glucose sensing (Navarro *et al.* 1996). This dependence on GK has



**Figure 6** Effects of 20 mM glucose on GK activities in LH were not produced by hypertonic change on the cells. The hypothalamic slice-explant cultures were incubated for 3 h in a medium that contained the indicated concentration of glucose and sucrose. In all cases, the final concentrations of sugars in the extracellular medium were 20 mM. VMH and LH nuclei were dissected from the hypothalamic slices after the incubation period and glucose phosphorylating activities were determined in soluble fractions from these areas. Values of GK activities are given as means  $\pm$  S.E.M. for at least three independent experiments repeated in duplicate. \*\*\* $P \leq 0.001$  (LH versus VMH).

recently been confirmed by demonstrating that transfected cultured VMH neurons with GK siRNA almost completely abolished neuron glucose sensing, and that pharmacological activation of GK increased the sensitivity of glucose-sensing neurons to glucose (Kang *et al.* 2006).

Neither the promoter activity of the GK gene in transfected GT1-7 cells nor the endogenous GK gene expression were modified by the action of different concentrations of glucose or GLP-1, LEP, or NPY in the extracellular space. However, GK enzyme activities were modified by these molecules, suggesting that alterations may be induced through short-term processes rather than by longer-term mechanisms involved in GK gene expression. In this sense, we cannot discard other short-term effects, i.e. the interactions of GK and GKRP present in the hypothalamus of rat and human brain (Alvarez *et al.* 2002, 2005) or GK-PFK2 interaction as occurs in liver and  $\beta$ -cells (Massa *et al.* 2004, Baltrusch & Tiedge 2006).

In previous reports, we described the presence of GK mRNA and protein in rat (Navarro *et al.* 1996, Roncero *et al.* 2000) and human (Roncero *et al.* 2004) brains, with similar kinetic properties as the enzyme from liver and pancreatic islets. In this work, we present experimental evidence of the existence of GK protein and activities in VMH and LH areas, which permits comparative analyses between different hypothalamic areas, such as we observed in the VMH and

LH in response to glucose. Whereas in GT1-7 cells, glucose did not modify the expression of GK gene and GK activity at the time studied, in the VMH there was a tendency for GK activities to increase as glucose rose in the extracellular media and high-GK activity was found at lower glucose concentrations in the LH. These findings could reflect a different type of behavior for the GE and GI neurons located in the VMH and the LH, when challenged by different concentrations of glucose. This would be in agreement with the tendency of GK activity to increase with rises in glucose in the range of 0.5–20 mM that we found in the VMH, while neurons in LH were not excited by the higher concentrations of glucose but by lower concentrations of this hexose, as happens with GK activities. The observed changes in GK activity in response to glucose in certain hypothalamic areas suggest that such activity would not only be tissue-specific but also even cell-specific in defined brain areas.

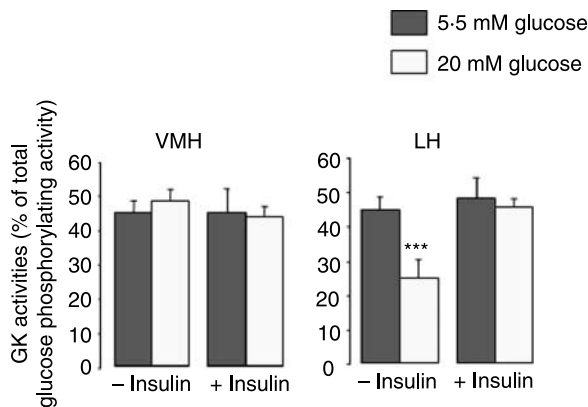
Since the concentrations of extracellular glucose in the brain seem to be lower than those in blood (from 0.5 to 4.5 mM; Silver & Erecinska 1994), we used a broad range of glucose concentrations from 0.05 to 20 mM in order to get general information of the effects of glucose on GK activities. We did observe effects of glucose not only within this range but also at 20 mM glucose; this could be explained in terms of the presence of neurons that are stimulated by an increase from 5 to 20 mM glucose (Fioramonti *et al.* 2004). Obviously, 20 mM glucose can be considered as a pharmacological concentration, but these levels may be found in the blood of uncontrolled diabetic patients and from a pathophysiological point of view the results obtained under this circumstance may be of interest. Interestingly, GK activities were significantly lower in LH than in VMH at 20 mM, but this effect was reversed by the presence of INS. This distinctive response observed in LH at 20 mM glucose may be related to different functional activities of these two areas, rather than to other kind of effects. Thus, the effects were induced by D-glucose but not by L-glucose, and the changes in total glucose-phosphorylating activities were due to modifications in GK but not in HKs. In addition, the hypertonic effect of high-glucose concentrations used was not responsible for the observed modifications in GK since similar concentrations of sucrose did not modify the enzyme activities.

Since we have previously proposed that GK may be a glucose sensor in brain (Navarro *et al.* 1996) implicated in the control of food ingestion, and orexigenic and anorexigenic peptides play important roles on feeding behavior, we tested

**Table 2** Glucokinase activities as percentage of total glucose phosphorylating activity in hypothalamic slices (ventromedial hypothalamus, VMH and lateral hypothalamus, LH areas) incubated in the presence of 5.5 mM glucose with or without (–) peptides. Values are means  $\pm$  S.E.M. (*n*)

	–	GLP-1	LEP	OREX	INS	NPY
VMH	54.9 $\pm$ 6.6 (8)	51.4 $\pm$ 6.4 (7)	40.9 $\pm$ 10.8 (4)	36.7 $\pm$ 5.7 (4)	50.2 $\pm$ 8.5 (6)	37.8 $\pm$ 26.4 (2)
LH	53.3 $\pm$ 5.6 (8)	47.9 $\pm$ 6.0 (7)	48.7 $\pm$ 7.0 (4)	43.9 $\pm$ 11.0 (4)	52.6 $\pm$ 8.3 (6)	35.0 $\pm$ 6.5 (4)*

\* $P < 0.05$  compared with (–) condition. GLP-1, glucagon-like peptide 1; LEP, leptin; OREX, orexin-B; INS, insulin; NPY, neuropeptide Y.



**Figure 7** Effect of insulin and 20 mM glucose on GK activities in LH. The hypothalamic slice-explant cultures were incubated for 3 h in a medium that contained 5.5 or 20 mM glucose in the presence or absence of 10 nM insulin. VMH and LH nuclei were dissected from the hypothalamic slices after the incubation period and glucose phosphorylating activities were determined in soluble fractions from these areas. Values of GK activities are given as means  $\pm$  S.E.M. for at least three independent experiments repeated in duplicate. \*\*\* $P \leq 0.001$  (5.5 mM glucose versus 20 mM glucose).

the effects of these peptides on *GK* gene expression and *GK* enzyme activities. Although these peptides did not modify the transcriptional activity of the *GK* gene in GT1-7 cells except for a significant negative effect of INS, these peptides did alter the *GK* activities in GT1-7 cells and in the VMH and LH. In this way, we have found in humans after the evaluation of cerebral glucose metabolism in control subjects by positron emission tomography, using 2-[F-18]deoxy-D-glucose, that GLP-1 modified cerebral glucose metabolism in hypothalamus and brainstem (Alvarez *et al.* 2005).

The expression of hepatic (L1) and the pancreatic islet (B1) functional *GK* isoforms is controlled in a different way since liver *GK* is regulated by INS, whereas the pancreatic enzyme seems to be controlled posttranslationally by glucose levels (Bedoya *et al.* 1986). However, in recent years it has been reported (Leibiger *et al.* 2001, Da Silva Xavier *et al.* 2004) that INS positively regulates *GK* gene transcription in pancreatic  $\beta$  cells. Additionally we have found a similar RNA splicing of the *GK* gene product in rat hypothalamus (Roncero *et al.* 2000) and pancreatic islets; the mRNA that codes for the B<sub>1</sub> isoform was the most abundant at both locations, which poses the question of whether the control of *GK* gene expression and enzyme activity in the hypothalamus would be modulated by INS and/or by glucose. At the time studied, our data revealed that changes in glucose levels were unable to stimulate the transcriptional activity of the *GK* gene in GT1-7 cells, although INS was the only peptide assayed that inhibited both *GK* gene transcription and enzyme activity, indicating that this isoform may differ as compared with those of liver or pancreatic origin.

In conclusion, our findings indicate that in most of the cases modifications of *GK* occur at the enzyme activity level rather than in transcriptional expression, suggesting that if *GK* acts as

a glucose sensor involved in feeding behavior it requires changes in short rather than longer periods of time, i.e. alterations in *GK* activity rather than in gene expression. Moreover, the distinctive pattern of *GK* activities of ventromedial and lateral hypothalamic areas would be conditioned by the action of GE and GI neurons in such locations and their potential role in glucose sensing. Finally, the effect of orexigenic (NPY and orexin) and anorexigenic (LEP, GLP-1 and INS) peptides on *GK* activities raise further questions about the action of these molecules in glucose sensing and nutrient homeostasis in the brain.

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