Original Article

A Glucose Sensor Role for Glucokinase in Anterior Pituitary Cells

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Enzymatic activity of glucokinase was demonstrated, quantitated, and characterized kinetically in rat and mouse pituitary extracts using a highly specific and sensitive spectrometric assay. A previously proposed hypothesis that the glucokinase gene might be expressed in the pituitary corticotrophic cells was therefore reexamined using mRNA in situ hybridization and immunohistochemical techniques. No evidence was found that corticotrophs are glucokinase positive, and the identity of glucokinase-expressing cells remains to be determined. The findings do, however, suggest a novel hypothesis that a critical subgroup of anterior pituitary cells might function as glucose sensor cells and that direct fuel regulation of such cells may modify the classical indirect neuroendocrine pathways that are known to control hormone secretion from anterior pituitary cells. *Diabetes* 55:1923–1929, 2006

Glucose homeostasis is maintained by the balanced secretion and action of insulin on one side and glucagon, epinephrine, cortisol, and growth hormone on the other (1,2). The secretion of these hormones is governed in turn by glucose sensor cells, most often with glucokinase as the glucose sensor (3,4). Glucose sensing can be either direct as in the case of the insulin-producing β-cells or indirect as in the case of the epinephrine-releasing chromaffine cells, which are stimulated by nicotinic cholinergic neurons that are controlled by glucose sensors of the central and autonomic nervous systems (2,4,5). Glucose regulation of glucagon, cortisol, and growth hormone is also believed to be indirect. The glucagon-producing pancreatic α-cells contain glucokinase (6,7), but the overriding glucose control of glucagon secretion is probably exerted indirectly via β-cell–derived factors. The other counterregulatory hormones ACTH/cortisol and growth hormone are thought to be regulated by hypothalamic releasing and inhibitory hormones produced by diverse groups of glucose-sensing neurons (2). There are, however, reports from more than a decade ago that explored the possibility (discounted in the end) that pituitary cells themselves might express glucokinase (8–10). We have reinvestigated this physiologically attractive possibility using greatly improved analytical methods for detecting glucokinase and have indeed found evidence for glucokinase-based glucose sensing in cells of the anterior pituitary. Although we provide evidence casting strong doubt on our initial working hypothesis that the corticotrophic cells might be the glucose sensors and remain in the dark about the identity of glucokinase-positive pituitary cells, our observations promise to contribute significantly to the concept, still unfolding, that a broad network of glucokinase-expressing cells, including pituitary cells, governs glucose homeostasis.

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Received for publication 1 February 2006 and accepted in revised form 14 April 2006.

GKA, glucokinase activator; GST, glutathione S-transferase; POMC, proopiomelanocortin.

DOI: 10.2337/db06-0151

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RESEARCH DESIGN AND METHODS

Tissue and cell preparations. Pituitary glands and liver were dissected from randomly fed male and female C57BL6 mice or male Wistar rats anesthetized with pentobarbital. Tissues were weighed and then stored frozen at −80°C until used. Rat pancreatic islets were isolated with a collagenase procedure as described and also stored frozen until used (11). AtT20, GH3, and GLUTag cells were obtained from various sources: AtT20 and GH3 cells were from the American *Type Culture* Collection (Manassas, VA) and GLUTag cells were a gift from Dr. Daniel Drucker (Toronto, Canada). They were cultured as follows: AtT20 and GH3 cells were cultured in Ham's F12 medium with 2 mmol/l L-glutamine, 10 mmol/l glucose, 2.5% FCS, and 10% horse serum; GLUTag cells were cultured in Dulbecco's modified Eagle's medium with 24 mmol/l glucose and 10% FCS. Both media had penicillin/streptomycin added. They were cultured under 95% O_2/5% CO_2 until they reached ~75% confluency when they were harvested and stored frozen until used. Frozen tissues and cells were subjected to several freeze/thaw cycles and were homogenized in 25 mmol/l Hepes buffer (pH 7.4) containing 150 mmol/l KCl, 2 mmol/l dithiothreitol, and 1 mmol/l EDTA. The homogenates were briefly centrifuged at low speed to remove coarse particulate matter that interfered with spectrometry. These crude extracts were used right after preparing them to determine glucokinase activity. A small aliquot was saved frozen for measuring protein by the BioRad method with BSA as standard (Hercules, CA).

Spectrometric glucokinase assay. A pyridine nucleotide-coupled assay was used, and the final product NADH was measured spectrophotometrically with a 96-well plate reader (Molecular Devices, Sunnyvale, CA). The assay volume was 120 microl/well. The product of the glucokinase reaction, glucose-6-P, was oxidized by the NAD-specific glucose-6-P dehydrogenase from Leuconostoc Mesenteroides. The composition of the assay reagent was as follows: 100 mmol/l Hepes buffer (pH 7.4), 5 mmol/l ATP (sodium salt), 6 mmol/l $MgCl_2$, 0.1% BSA, 150 mmol/l KCl, 1 mmol/l dithiothreitol, 45 μmol/l 5-thio-D-glucose-6-P ([12] to inhibit other interfering hexokinases, most importantly hexokinases I), 1 mmol/l 3-O-methyl-*N*-acetylglucosamine ([13] to inhibit *N*-acetylglucosamine kinase), and different glucose concentrations (0, 0.5, 1.5, 3.0, 6.0, 20, and 60 mmol/l), both in the presence and absence of 30 μmol/l of a glucokinase activator (GKA) (14). The medium contained 2.5 IU/ml glucose-6-P dehydrogenase. The analysis of each tissue sample also included human wild-type glutathione S-transferase (GST)-glucokinase fusion protein (15,16) as biological standard at 0.2 ng/well and a mixing experiment (0.2 ng/well

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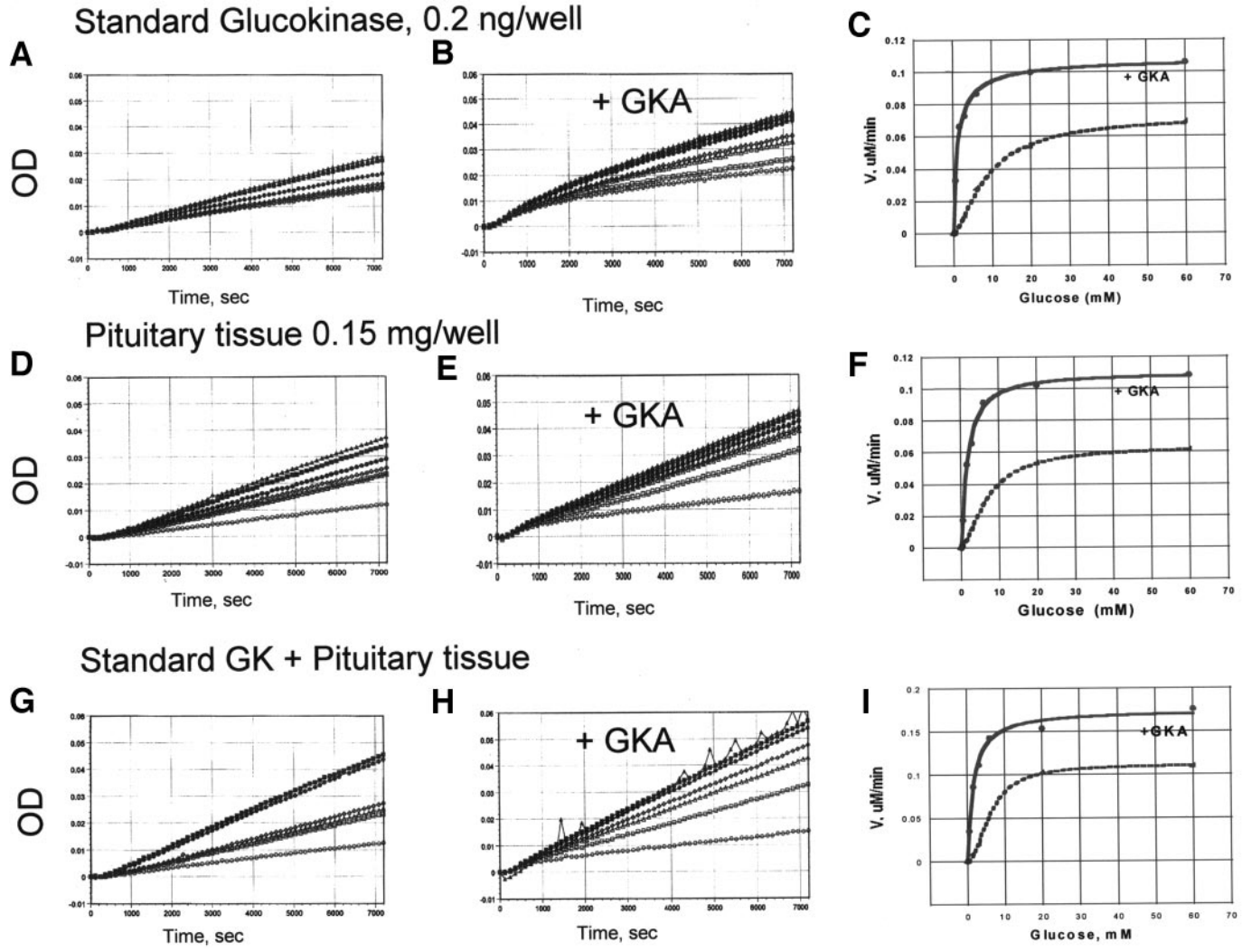

FIG. 1. Results of a typical glucokinase assay using crude rat pituitary extract. Three-hour progress curves of glucose phosphorylation at increasing substrate concentrations ranging from 0 to 60 mmol/l glucose for an optimal amount of crude pituitary extract (0.15 mg/well) are shown (*D* and *E*). Wild-type GST-glucokinase fusion protein (0.2 ng/well) served as a biochemically defined standard (*A* and *B*) and was also used in mixing experiments to assess recovery (*G* and *H*). The measurements were made in the absence and presence of 30 mmol/l GKA as indicated. *C*, *F*, and *I* show the concentration dependency curves in the presence and absence of the allosteric activator. Note the clearly sigmoidal nature of the curves in the absence of the activator.

GST-glucokinase plus tissue extract) to assess recovery or the possibility of interference. Assays were carried out in the presence of near-saturating GKA (RO-0274375) to guarantee specificity of analysis. Altogether, 49 data points were generated for the glucokinase assay of every sample (see Fig. 1 as an example for this test). Calculations were done as previously described and resulted in information on V_{max}, glucose $S_{0.5}$, and the Hill coefficient (nH), both in the presence and absence of the GKA (17).

Glucokinase mRNA measurements via in situ RNA hybridization and expression microarrays. A 433-bp mouse growth hormone fragment, a 588-bp glucokinase fragment, and a 491- or 492-bp proopiomelanocortin (POMC) fragment were cloned into pCR2.1 (Invitrogen, Sunnivale, CA). The digoxigenin-labeled riboprobes were synthesized as follows. *1*) Growth hormone and glucokinase fragments were linearized with *Bam*HI (Roche, Basel, Switzerland) and transcribed with T7 RNA polymerase (Promega, Madison, Wisconsin). *2*) The POMC plasmid was linearized with *Hind*III (Roche) and transcribed with T7 RNA polymerase. Primer sequences used to amplify DNA fragments are available upon request. Wild-type mouse pituitary was fixed in 4% paraformaldehyde and stored in 70% ethanol before being embedded in paraffin and sectioned at 5 μm. RNA in situ hybridization was performed according to the Wilkinson protocol (18). The tissue distribution of mRNA-encoding glucokinase and its regulatory protein in the mouse using expression microarrays was studied in male C5BL6J mice (8–12 weeks of age). Total RNA was extracted ex vivo from mouse pituitary, brain, adrenal gland, liver, skeletal muscle, epididymal adipose tissue, lung, kidney, and spleen using TRIzol Reagent according to the manufacturer's protocol (Gibco, Carlsbad, CA) followed by a clean-up procedure with RNeasy columns (Qiagen, Cologne, Germany). In addition, total RNA from freshly isolated mouse pancreatic islets and pancreatic acini, hand selected under a dissecting microscope in ice-cold media, was extracted using the Absolutely RNA microprep from Stratagene (La Jolla, CA). Total RNA quantity and quality was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the 2100 Bioanalyzer (Agilent, Waldbronn, Germany), respectively. Total RNA profiles of all tested samples were similar with sharp 18S and 28S rRNA peaks on a flat baseline. RNA quantification was performed using Affymetrix Mouse 430 2.0 expression microarrays (Affymetrix, Santa Clara, CA). Biotin-labeled cRNA was labeled overnight and then fragmented for 35 min at 94°C; concentration and quality of labeled cRNA was measured, respectively, with the NanoDrop ND-1000 and Bioanalyzer 2100. Fragmented cRNA was hybridized onto the 430 2.0 arrays during 16 h at 45°C, followed by washing/staining in the Fluidics Station (Affymetrix), and quantitated by scanning using a 3000 GeneScanner. Raw data were analyzed using GCOS software. Signal intensities were scaled using the global scaling method, using 150 as target intensity value. For all tissues, at least three independent biological replicates were studied; islets and pituitaries were studied with n = 4–5 per condition.

Immunohistochemistry. Rat pituitaries were fixed in 4% paraformaldehyde at 4°C. Specimens were mounted in paraffin, and serial 5-μ sections were made. Both peroxidase and fluorescent labeling followed established procedures. Primary antisera included glucokinase (sheep anti-glucokinase; a gift from M. Magnuson) and ACTH (mouse monoclonal; Abcam, Cambridge, MA).

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Secondary antibodies included donkey anti-sheep conjugated with rhodamine red and anti-mouse IgG conjugated with fluorescein isothiocyanate and were obtained from Jackson Immunoresearch Laboratories, West Grove, PA. Images were acquired using a Nikon Eclipse E600 with a digital Hamamatsu Photonic System.

RESULTS

Enzymological evidence for the presence of glucokinase in the pituitary. A sensitive and specific spectrometric assay was developed affording the opportunity to reassess the possibility that the low-affinity glucose sensor enzyme glucokinase is present in pituitary cells. The advance was facilitated by two factors: *1*) the availability of powerful and specific inhibitors of high-affinity hexokinases (i.e., 45 μmol/l of 5-thioglucose-6-phosphate) and of *N*-acetyl-glucosamine kinase (i.e., 1.0 mmol/l of 3-O-methyl-*N*-acetyl-glucosamine), enzymes that had interfered in previous measurements (12,13); and *2*) the discovery of novel, highly specific allosteric activators of GKAs, which provide the clear pharmacological proof that glucokinase is being measured in the present study (14). Figure 1 illustrates this assay as applied to crude rat pituitary extracts. Reaction progress curves and glucose dependency plots for recombinant human GST-glucokinase fusion protein, pituitary extract, and the combination of the two (both in the presence and absence of GKAs) demonstrate clearly that the enzyme is present in the pituitary. The sensitivity and reproducibility of the measurements are sufficient to allow the reliable calculation of essential kinetic constants, both in the presence and absence of GKAs (Table 1). We observed a glucose $S_{0.5}$ of ~8.0 mmol/l, a Hill coefficient of >1.4, and that GKAs lower the glucose $S_{0.5}$ by a factor of ~5 and in most instances increase the V_{max}. These biochemical data allow the identification of the glucose phosphorylating activity recorded here as glucokinase. Results of surveys using crude extracts of liver, isolated pancreatic islets, AtT20, and GH3 cell lines of pituitary origin and of the enteroendocrine GLUTag cells are provided for comparison. Not surprisingly, there are marked differences in the V_{max} values, yet the kinetic constants and the GKA activations were comparable in all cases as expected. It is noteworthy that the GH3 cell of the mammosomatotroph lineage had no detectable glucokinase (not shown).

Glucokinase mRNA is expressed in mouse and rat pituitary. Glucokinase mRNA levels in mouse pituitary were comparable to those found in liver and pancreatic islet extracts (Fig. 2). Assays of whole brain, adrenal gland, exocrine pancreas acini, muscle, adipose tissue, lung, kidney, and spleen using expression microarrays indicated that glucokinase was not expressed in these tissues. Glucokinase regulatory protein mRNA was detected only in the liver. Starvation for 20- or 2-h refeeding after 20-h starvation showed no changes of pancreatic islet or pituitary glucokinase mRNA (data not shown). In situ hybridization with a probe for glucokinase mRNA yielded positive results in the form of a punctate pattern confined to the anterior pituitary lobe (Fig. 3). Evidence for expression of ACTH mRNA was found in the intermediate and anterior lobes, whereas growth hormone mRNA was demonstrated only in the anterior lobe. The patterns of distribution for ACTH mRNA and growth hormone mRNA are compatible with the known cell densities and locations of POMC and growth hormone–expressing cells of the hypophysis (19). The punctate pattern of expression in the anterior pituitary suggests that the enzyme is present in a

TABLE 1
Similarities of kinetic and pharmacological characteristics of liver, pancreatic islets, pituitary, AtT20 cells, and GLUTag cells

Tissue	*n*	V_{max} (nmol · mg tissue^{-1} · h^{-1})		Glucose $S_{0.5}$ (mmol/l)		nH		% recovery		Relative activity (islet glucokinase = 1)	
		Basal	+GKA	Basal	+GKA	Basal	+GKA	Basal	+GKA	Basal	+GKA
Mouse liver	5	215 ± 23.5	283 ± 31.5	9.64 ± 0.89	1.35 ± 0.09	1.57 ± 0.03	1.14 ± 0.02	96.0 ± 0.61	99.0 ± 1.70	9.92	12.1
Freshly isolated rat islets	9	21.7 ± 2.22	23.4 ± 1.76	8.10 ± 0.80	0.93 ± 0.09	1.38 ± 0.06	1.41 ± 0.18	88.9 ± 4.49	88.9 ± 2.35	1	1
Pituitary (rat)	5	3.30 ± 0.18	4.90 ± 0.26	8.93 ± 2.01	1.48 ± 0.35	1.44 ± 0.09	0.98 ± 0.06	81.0 ± 8.81	80.8 ± 2.63	0.15	0.21
Pituitary (mouse)*	2	4.09 ± 0.09	4.62 ± 0.09	7.98 ± 0.03	1.52 ± 0.23	1.86 ± 0.15	1.34 ± 0.08	97 ± 1.41	124 ± 24	0.19	0.2
AtT-20 cells	8	0.8 ± 0.07	1.1 ± 0.08	7.78 ± 0.33	0.85 ± 0.06	1.72 ± 0.11	1.04 ± 0.04	—	—	0.04	0.05
GLUTag cells	9	65.9 ± 34	74.5 ± 40.1	8.8 ± 3.5	1.5 ± 0.5	1.4 ± 0.5	1.3 ± 0.5	—	—	3.04	3.18

Data are $n \pm$ SE, unless otherwise indicated. *Five mouse pituitaries were pooled for each of the two analyses. Results were obtained by assays conducted in the absence of GKAs and also with near-saturating 30 μmol/l of a GKA. Note that all V_{max} values are expressed in terms of nanomole of product generated per milligram of tissue per hour. This uniformity of expression was achieved by using an interconversion factor of 7.0 in cases where protein measurements rather than tissue weights were available. Recovery was calculated by distributing loss equally between tissue sample and recombinant glucokinase standard.

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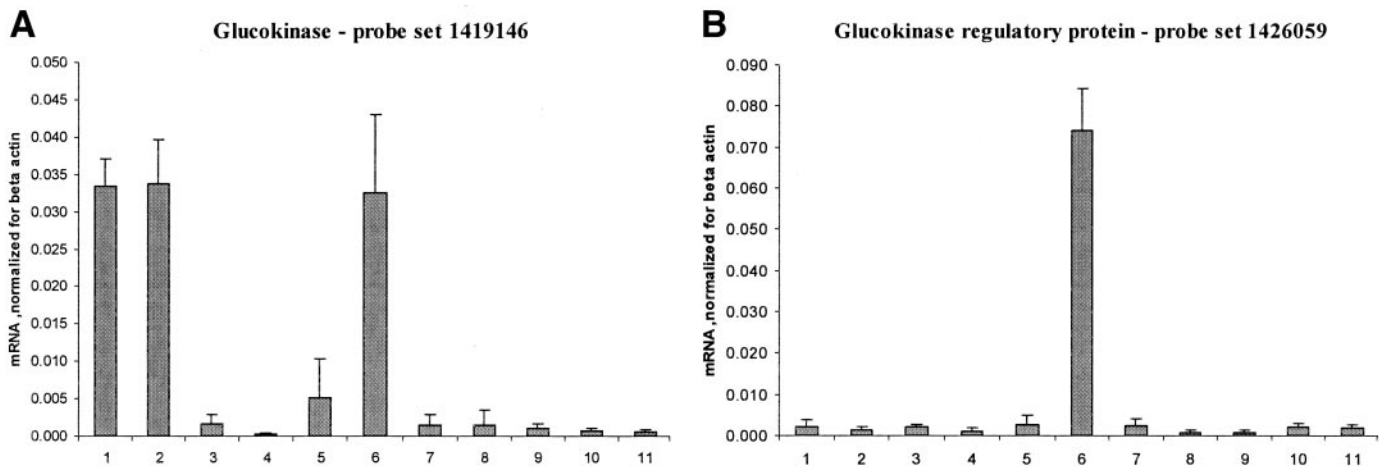

FIG. 2. Quantification of relative mRNA abundance of glucokinase and glucokinase regulatory protein in various tissues using expression microarrays. mRNA signals were normalized for β-actin mRNA in the same sample. The results presented in *A* and *B* were obtained from the following tissues: *1*) islets of Langerhans (pancreas), *2*) pituitary, *3*) adrenal gland, *4*) whole brain, *5*) acinar pancreas, *6*) liver, *7*) skeletal muscle, *8*) adipose tissue, *9*) lung, *10*) kidney, and *11*) spleen.

unique cell type, which contributes ≤10% to the total cell mass of the anterior lobe. It is not possible to draw any conclusions regarding the endocrine identity of these glucokinase-expressing cells from the in situ hybridization results and the immunohistochemical data described below if one considers the possibility that only a fraction of a particular cell type might express glucokinase.

Immunohistochemistry of glucokinase-expressing cells in the anterior pituitary. To identify glucokinase-positive cells, we have begun to study the histochemistry of the pituitary in a systematic fashion. Using peroxidase-based immunohistochemistry, glucokinase-positive cells were found distributed in a random pattern throughout the anterior pituitary (Fig. 4*A*). Remarkably, posterior and intermediate lobe cells showed only faint and uniform staining, which was interpreted as unspecific background, consistent with the in situ hybridization data presented in Fig. 3. It is estimated that maximally 10% of the anterior pituitary cells are glucokinase positive, in good agreement with our results from in situ hybridization. The glucokinase cells are relatively large and pear shaped. Doublet and triplet groups can be made out in several sections, an arrangement that indicates that contiguous glucokinase cell networks of various sizes might exist. However, as alluded to above, comparing the results of dye histochemistry, in situ hybridization, and immunohistochemistry is not sufficient for identifying the endocrine or any other nature of glucokinase-positive cells. To determine the identity of the glucokinase cells, we focused initially on the possibility that corticotrophs might be the glucose sensor cells because previous reports had pointed in that direction (4,8). An immunohistochemical stain for ACTH using the peroxidase method showed uniform dark staining in the intermediate lobe (not shown) and of individual cells scattered throughout the anterior portion of the gland (Fig. 4*B*). The shape of these ACTH cells differs clearly from that of the glucokinase cells. They have a distinct multiangular shape with several more or less sharply defined corners. Furthermore, there appear to be more ACTH cells than glucokinase-positive cells, consistent with the distribution of respective RNA expression patterns as demonstrated by in situ hybridization (Fig. 3). Double staining for ACTH and glucokinase using fluorescent antibodies showed no evidence for coincidence of green (ACTH) and red (glucokinase) staining, strongly arguing against glucokinase expression in the corticotroph (Fig. 4*C*). Careful inspection of the fluorescent antibody–stained sections at higher magnification (Figs. *D-F*) shows the same difference in cell shape that was noticed in the peroxidase-based stains of glucokinase- and ACTH-positive cells, again confirming that the ACTH cells do not contain demonstrable glucokinase protein.

DISCUSSION

The results of the present study (reported in an abstract by Zelent et al. [20] and briefly discussed by Matschinsky et al. [4]) clearly demonstrate that anterior hypophyseal cells express significant amounts of the glucose sensor enzyme glucokinase. The observed values of the kinetic constants and the characteristic responsiveness to allosteric GKAs leave no doubt that glucokinase is being measured. The actual cellular rate of glucokinase-mediated glucose phosphorylation measurable in crude pituitary extracts is by extrapolation quantitatively comparable to that of pancreatic islets, considering that ~10% of pituitary tissue is glucokinase positive. The present results of glucokinase mRNA measurements also support the interpretation that pituitary cells express glucokinase and show a clear distinction to the situation in the liver where glucokinase regulatory protein plays a critical role in the regulation of the enzyme (21,22). In that respect and also by the absence of a fasting/refeeding response of glucokinase mRNA, glucokinase-expressing pituitary cells resemble pancreatic islet cells (23,24). It is, however, noteworthy that the relative concentrations of mRNA and enzyme levels of the different tissues examined here do lack comparable quantitative relationships, suggesting fundamental differences in RNA and protein biosynthesis and/or degradation. Previous molecular genetic studies of glucokinase mRNA processing by pituitary tissue had concluded that differential splicing resulted in a message not suitable to be translated and folded into a catalytically competent enzyme (9,10). It is entirely possible that the bulk of pituitary cell glucokinase mRNA is differentially spliced, as these earlier studies seemed to show, and that only a minor fraction is processed in the orthodox manner, resulting in a functional enzyme only in a relatively small subpopula-

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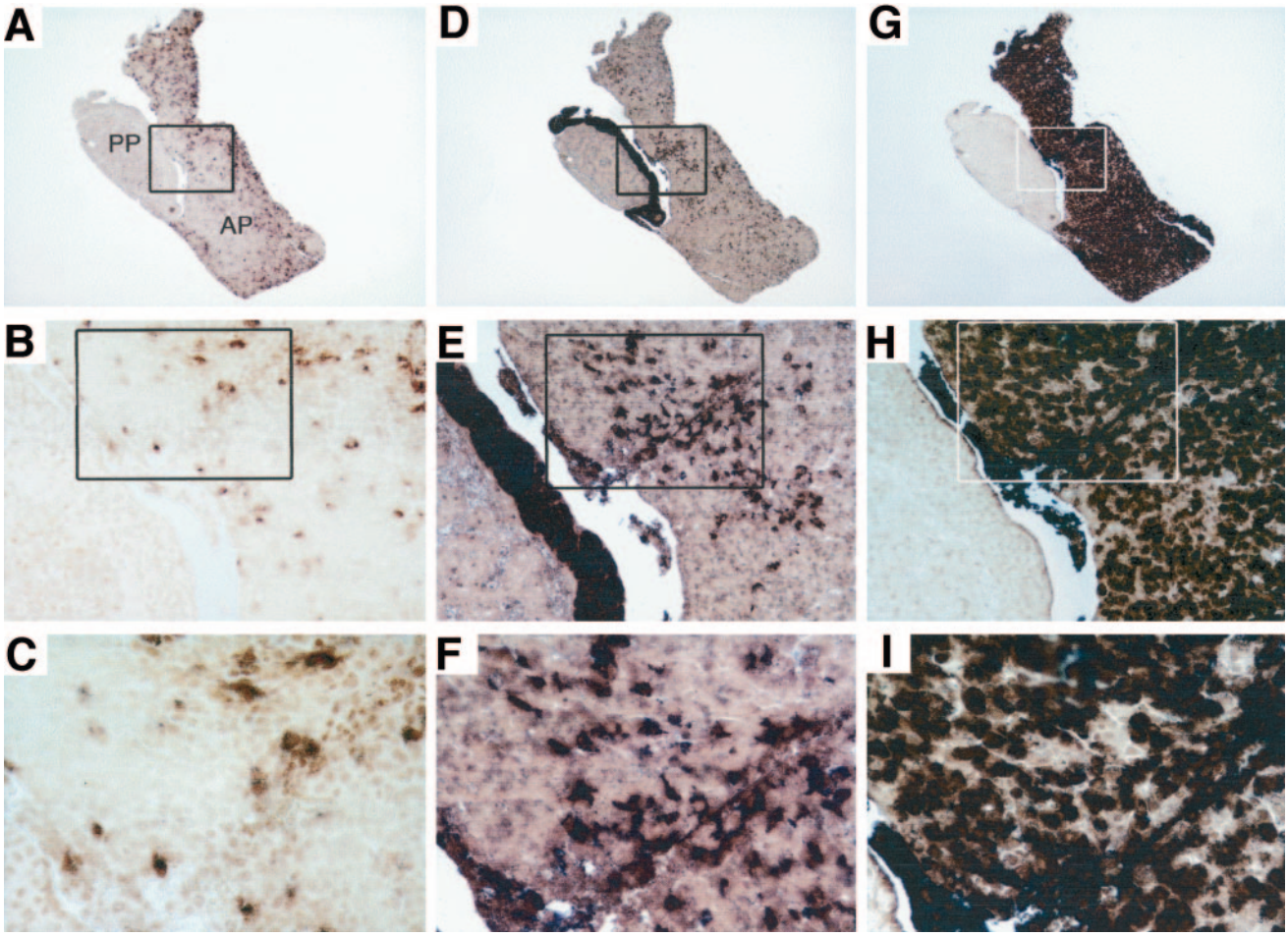

FIG. 3. Cellular distribution pattern of glucokinase mRNA in mouse pituitary as related to expression of ACTH/POMC and growth hormone (GH). RNA in situ hybridization studies are shown for glucokinase (*A–C*), ACTH/POMC (*D–F*), or GH (*G–I*). Images in *A*, *D*, and *G* were taken at 4×, in *B*, *E*, and *H* at 20×, and in *C*, *F*, and *I* at 40× magnification. Boxes indicate areas shown at higher magnification.

tion of specialized glucose sensor cells. The identity of these glucokinase-positive cells has eluded us thus far. It is clear that this population is relatively small and seemingly randomly distributed throughout the anterior lobe. We have, however, established that corticotrophs, which we had considered as prime candidates for pituitary glucose sensor cells, do not contain significant levels of the enzyme. This result was surprising to us considering earlier reports providing some evidence that POMC cells might express glucokinase (4,8,9) and the present finding that corticotroph-derived AtT-20 cells contained some authentic glucokinase activity. Since there are at least eight distinct cell types in the anterior pituitary gland, any speculation on the nature and role of the glucokinase-positive cell may seem premature. Still, assuming that the enzyme confers glucose sensitivity to this particular glucokinase-positive cell type, whatever it may be, a few implications of the present observations need to be considered. It will be critical to explore whether the glucokinase-positive pituitary cells have other biochemical characteristics that are found in the prototypical glucose-sensitive insulin or glucagon-secreting β- or α-cells or whether they represent a unique type of glucose sensor cell (4,25). It will be important, for example, to check what the nature of the glucose transporter(s) might be, whether the SUR-1/Kir-6.2 potassium channel complex is present or not and what the biochemical features of any calcium channels might be. It is possible that a unique glucose sensor cell exists in which glucose acts primarily by altering intracellular calcium storage rather than by modifying ion channels of the cell membrane as true for pancreatic β-cells and certain hypothalamic neurons (4,5). Our current general physiological understanding of the anterior pituitary indicates that pituitary cells are controlled entirely by the hypothalamic neuroendocrine system (i.e., by numerous classical stimulatory and inhibitory neurohormones) and by powerful feedback via cortisol, thyroid hormone, sex steroids, etc. (19). However, the present finding that a significant population of anterior pituitary cells have the capability to sense glucose would argue in favor of an additional regulatory pathway, namely for the existence of direct regulation of pituitary cells by blood glucose levels. Future research should therefore consider a paradigm modification and explore the possibility of dual control of pituitary cell function by the classical neuroendocrine factors and feedback on one hand and directly by glucose and perhaps other fuels including amino acids and free fatty acids on the other. The discovery of substantial levels of the glucokinase glucose sensor in anterior pituitary cells expands the complex network of glucokinase-expressing cells significantly by the inclusion of the endocrine master gland of the body, posing a considerable challenge to discover the cellular identity of these presumed glucose sensor cells

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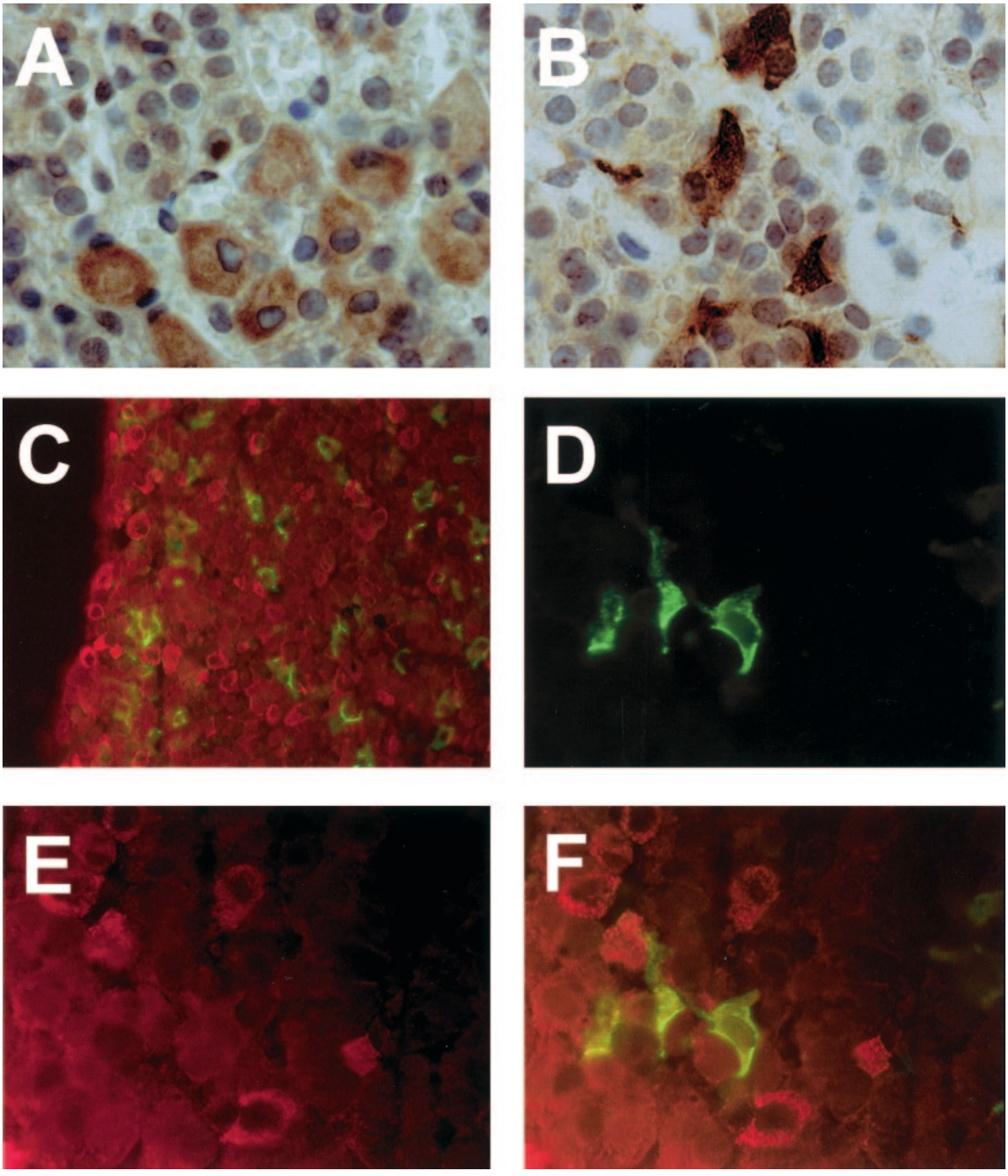

FIG. 4. Immunohistochemistry of glucokinase- and ACTH-expressing cells of the anterior pituitary lobe. The magnification of the images (40 or 100×) and the dilutions of the primary antibody (1:50 or 1:100) are given for each panel. *A* (100×): Immunoperoxidase stain for glucokinase (1:100). *B* (100×): Immunoperoxidase stain for ACTH (1:100). *C* (40×): Double staining with fluorescent antbodies against glucokinase (*red*; 1:50) and ACTH (*green*; 1:100) showing apparent lack of colabeling. *D–F* (100×): Fluorescent antibody stains of ACTH cells (*D*; 1:100), glucokinase-positive cells (*E*; 1:50), and fusion of *D* and *E* (*F*).

and explore physiological, pathological, and pharmacological ramifications that could arise from their existence.

ACKNOWLEDGMENTS

The work was supported by the National Institutes of Health (NIDDK 22122 to F.M.M. and 55342 to K.H.K.) and by grants of the Juvenile Diabetes Research Foundation (1-2002-801) and the University of Leuven (GOA/2004/11) to F.S.

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