Regulation of Gene Expression by Glucose in Pancreatic $\beta$-Cells (MIN6) via Insulin Secretion and Activation of Phosphatidylinositol 3’-Kinase*

Received for publication, July 24, 2000, and in revised form, August 22, 2000
Published, JBC Papers in Press, August 30, 2000, DOI 10.1074/jbc.M006597200

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Increases in glucose concentration control the transcription of the preproinsulin (PPI) gene and several other genes in the pancreatic islet $\beta$-cell. Although recent data have demonstrated that secreted insulin may regulate the PPI gene (Leibiger, I. B., Leibiger, B., Moede, T., and Berggren, P. O. (1998) Mol. Cell 1, 933–938), the role of insulin in the control of other $\beta$-cell genes is unexplored. To study the importance of insulin secretion in the regulation of the PPI and liver-type pyruvate kinase (L-PK) genes by glucose, we have used intranuclear microinjection of promoter-luciferase constructs into MIN6 $\beta$-cells and photon-counting imaging. The activity of each promoter was increased either by 30 (versus 3) mM glucose or by 1–20 mM insulin. These effects of insulin were not due to enhanced glucose metabolism since culture with the hormone had no impact on the stimulation of increases in intracellular ATP concentration caused by 30 mM glucose. Furthermore, the islet-specific glucokinase promoter and cellular glucokinase immunoreactivity were unaffected by 30 mM glucose or 20 mM insulin. Inhibition of insulin secretion with the Ca$^{2+}$ channel blocker verapamil, the ATP-sensitive K$^+$ channel opener diazoxide, or the $\alpha_1$-adrenergic agonist clonidine blocked the effects of glucose on L-PK gene transcription. Similarly, 30 mM glucose failed to induce the promoter after inhibition of phosphatidylinositol 3’-kinase activity with LY294002 and the expression of the promoter after inhibition of phosphatidylinositol 3,4,5-trisphosphate (PI3K) (Δp85) or the phosphoinositode 3’-phosphatase PTEN (phosphatase and tensin homologue). LY294002 also diminished the activation of the L-PK gene caused by inhibition of 5’-AMP-activated protein kinase with anti-5’-AMP-activated protein kinase α2 antibodies. Conversely, stimulation of insulin secretion with 13 mM KCl or 10 μM tolbutamide strongly activated the PPI and L-PK promoters. These data indicate that, in MIN6 $\beta$-cells, stimulation of insulin secretion is important for the activation by glucose of L-PK as well as the PPI promoter, but does not cause increases in glucokinase gene expression or glucose metabolism.

Elevated glucose concentrations stimulate the transcription of the preproinsulin (PPI) gene (1, 2) and several other genes in islet $\beta$-cells, including the liver-type pyruvate kinase (L-PK) gene (3), acetyl-CoA carboxylase I (4), GLUT2 (5), and a raft of other genes involved in insulin synthesis and release (6). However, the molecular mechanisms involved in the regulation of transcription by glucose are only partly understood (7, 8). Recent observations have suggested that the release of insulin may play an important part in the regulation of the preproinsulin gene by glucose, at least under certain conditions (9, 10). Consistent with this, increases in intracellular free Ca$^{2+}$ concentration ([Ca$^{2+}$]), which are important in the activation of insulin release, have been reported to be essential for regulation of PPI gene expression by glucose in some systems (11, 12). On the other hand, several studies have indicated that glucose can also regulate the PPI gene independently of insulin secretion (13–15). Furthermore, PPI gene expression appears to be largely unaltered after targeted disruption of the insulin receptor gene in the islet $\beta$-cell, at least in younger animals (16).

By contrast, the role of insulin secretion in the regulation of other glucose-sensitive islet $\beta$-cell genes is largely uninvestigated (8, 17). Elevations of glucose concentration enhance the expression of the L-PK gene in hepatocytes through activated transcription (18). This effect of glucose is dependent upon a cis-acting upstream region of the gene from nucleotides −183 to +10 with respect to the cap site (19), and a glucose response element has been mapped to a palindromic repeat of two E-boxes (CACGGG) located in the region at base pairs −170 to −150 with respect to the transcriptional start site (20). A similar region is also present in other glucose-responsive genes (8), including those encoding Spot14 (21), acetyl-CoA carboxylase I (22, 23), and fatty-acid synthase (24).

Phosphorylation of glucose appears to be essential for the transcriptional effects of the sugar on L-PK gene transcription in liver (7). Thus, glucose 6-phosphate and the pentose phosphate intermediate xylulose 5-phosphate (25) may be key signaling intermediates. The identity of the transcription factors mediating the transcriptional response is still debated, with evidence both for (26, 27) and against (28, 29) a role for the ubiquitous upstream stimulatory factor (USF1 and USF2). Recent data have also implicated sterol response element-binding protein-1c (SREBP-1c) (30) and other less well defined factors (31).

L-PK gene transcription is also strongly stimulated by insu-

* This work was supported by project grants from the Wellcome Trust, Diabetes UK (formerly the British Diabetes Association), the Medical Research Council (United Kingdom), and the Biotechnology and Biological Sciences Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: PPI, preproinsulin; L-PK, liver-type pyruvate kinase; [Ca$^{2+}$], intracellular free Ca$^{2+}$ concentration; USF, upstream stimulatory factor; SREBP-1c, sterol response element-binding protein-1c; GK, glucokinase (hexokinase type IV); P3K, phosphatidylinositol 3’-kinase; AMPK, 5’-AMP-activated protein kinase; KRB, Krebs-Ringer bicarbonate; CMV, cytomegalovirus; PI3K, phosphatidylinositol 3,4,5-trisphosphate.
lin in cultured liver cells. Under most experimental conditions, this effect requires elevated glucose concentrations and has been considered to be due mainly to up-regulation of the glucokinase (GK) gene (32) and thus increased intracellular concentrations of Glu-6-P or xylulose 5-phosphate (33, 34). However, insulin also activates the L-PK promoter after culture of hepatocytes from starved rats after constitutive expression of GK (35), indicating that an additional, glucose-independent mechanism of action of the hormone must exist.

Since high glucose concentrations stimulate islet GK gene expression relatively weakly (36, 37), if at all (3, 32), the inductive effect of glucose on L-PK gene transcription in islets and β-cell-derived INS-1 (3) and MIN6 (26, 38) cells has previously been attributed solely to an increase in the intracellular concentration of a glucose metabolite (e.g. Glu-6-P or xylulose 5-phosphate; see above). However, glucose also stimulates the release of stored insulin from the β-cell, so the potential exists for a para- or autocrine effect of the hormone on gene expression. We have therefore investigated the effects of added insulin on the PPI, L-PK, and GK gene promoters in the highly insulin-responsive MIN6 β-cell line (39). This system has enabled us to determine the relative importance of activated insulin secretion, glucose metabolism, and changes in [Ca²⁺], in the transcriptional regulation of the L-PK gene by glucose. Our results suggest that, under appropriate conditions, secretion of insulin and the activation of a signaling pathway dependent upon phosphatidylinositol 3-kinase (PI3K) largely explain the transcriptional effects of glucose on PPI and L-PK gene expression in this β-cell model.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium was from Sigma (Dorset, United Kingdom) or Life Technologies, Inc. (Paisley, UK). Beetle luciferin was from Promega, and coelenterazine was from Molecular Probes, Inc. Polyclonal anti-glucokinase antibody (sc-7908) was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). LY294002 was from Calbiochem-Novabiochem (Nottingham, UK). Porcine insulin was from Sigma. Other reagents were from Sigma or BDH.

**Plasmids**—pLPK-LucFP and p-150/LPK-LucFP contained nucleotides 1–183 to +10 and +148 to +183, respectively, of the rat L-PK promoter fused immediately upstream of humanized firefly luciferase cDNA (Promega plasmid pGL3-Basic™). Plasmid pINS-LucFP contained nucleotides 260 to −60 of the human insulin promoter fused upstream of a minimal herpes simplex thymidine kinase promoter and luciferase cDNA (40). The expression plasmid for Renilla luciferase (pRL-CMV) was purchased from Promega. pSRαΔv5 (provided by Prof. J. Tavare, University of Cambridge) contains the homeodomain-binding elements A1, A2, and A3, as well as the E-boxes E1 and E2 (51). pCMV-Luc® was provided by Prof. G. A. Rutter and E. K. Ainscow, submitted for publication.

**Antibodies**—Affinity-purified sheep antibodies raised against rat 5′-AMP-activated protein kinase (AMPK) α2 (42) were provided by Prof. D. G. Hardie (University of Dundee, Dundee, Scotland) and extensively dialyzed against microinjection buffer (2 mM Tris-HCl (pH 8.0) and 0.2 mM Na⁺/EDTA) before use.

**Cell Culture**—MIN6 β-cells (39) were used between passages 19 and 25 and grown in Dulbecco’s modified Eagle’s medium containing 15% (v/v) heat-inactivated fetal calf serum, 25 mM glucose, 5.4 mM KCl, 2 mM glutamine, 100 μM β-mercaptoethanol, 100 IU/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere at 37 °C with 5% CO₂ unless specified otherwise. Cells were transfected by medium containing 3 mM glucose 16 h prior to experiments. Secreted insulin was measured by radioimmunomassay (Linco Research Inc., St. Charles, MO) (43).

**Infection with Adenoviruses**—Cells were exposed to adenoviruses (30–100 viral particles/cell) for 2 h prior to culture for a further 24 h in the absence of added virus (43).

**Microinjection and Luciferase Imaging**—Intracellular microinjection of plasmids and antibodies was performed using an Eppendorf 5121/5246 micromanipulator (26, 44) at plasmid concentrations of 0.1 (LucFP®-based vectors) and 0.05 (pCMV-RL)-mg/ml and a total antibody concentration of 1–1.5 mg/ml (Bradford assay (45)) in 2 mM Tris-HCl, pH 8.0, 0.2 mM EDTA (38). Plasmid pSRαΔv5 or the corresponding empty vector (pSSRkα) was injected at 0.1 mg/ml. Individual experiments involved injection of 100–200 separate cells/condition, with an efficacy of 10–20% productive infection as assessed by expression of Renilla reniformis luciferase activity. Cells were imaged 6 h after microinjection and cultured under the conditions described above. Photocounting imaging of firefly and Renilla reniformis luciferase activities was performed in single living cells using an Olympus IX-70 inverted microscope (>10 air objective, 0.4 NA) and an intensified charge-coupled device camera (Photon, East Sussex, UK) as described in detail previously (26, 46).

**Measurement of [Ca²⁺], and Insulin Secretion**—Changes in [Ca²⁺], were measured at 37 °C with entrapped fura-2 (43, 47) using a Leica DM-IRBI inverted microscope (>40 objective) and a Hamamatsu C4742-995 charge-coupled device camera driven by OpenLabTM software (Improvement, Coventry, UK) (43, 48). Cells were loaded for 40 min in modified Krebs-Ringer bicarbonate (KRB) buffer (125 mM NaCl, 3.5 mM KCl, 1.5 mM CaCl₂, 0.5 mM MgSO₄, 0.5 mM KH₂PO₄, 2.5 mM NaHCO₃, and 10 mM NaH₂PO₄ (pH 7.4) initially containing 3 mM glucose and equilibrated with 95.5% O₂/CO₂ supplemented with 5 μM fura-2/AM (Sigma) and 0.1% Pluronic F-127 (BASF, Mount Olive, NJ). Insulin released into the culture medium was assayed by radioimmunoassay (Linco Research Inc.) (43).

**Intracellular ATP Concentration Imaging with Recombinant Expressed Luciferase**—Cytosolic luciferase (49) was expressed using an adenoviral vector (50) under constitutive (CMV) promoter control (Ad-CMVLuc®®) and monitored by real-time photon-counting imaging during perfusion in KRB buffer (37 °C) (43, 49).

**Statistical Analysis**—Data are given as means ± S.E. of three to five individual experiments. Comparisons between means were performed using one-tailed Student’s t test for paired data with Microsoft Excel™.

**RESULTS**

**Regulation of the PPI Promoter by Glucose and Exogenous Insulin in Single MIN6 β-Cells**—The activity of the human PPI promoter (base pairs −260 to −60 with respect to the transcriptional start site) (40) was measured by single cell luminescence assay (46) after microinjection of a firefly luciferase reporter construct. This promoter region contains the homeodomains—binding elements A1, A2, and A3, as well as the E-boxes E1 and E2 (51). PPI promoter activity was normalized to that of the CMV immediate-early gene promoter, linked to the expression of R. reniformis luciferase (26, 46, 52). In a previous report (14), we described conditions under which the PPI promoter was regulated in single MIN6 β-cells by glucose, independently of secreted insulin. In the present study, we observed that, by culturing MIN6 cells at (a) a lower passage number (passages 19–25) and (b) a lower density (thus decreasing the contribution of secreted insulin at low glucose concentrations), the PPI promoter was up-regulated more strongly (5–10-fold versus 1.5–2-fold in our previous report (14)) by 30 mM glucose and to a similar extent by 20 mM insulin (Fig. 1). The effect of 30 mM glucose was strongly inhibited by the l-type Ca²⁺ channel inhibitor verapamil and the type I PI3K inhibitor LY294002 (38). The effects of 30 mM glucose were also mimicked by low concentrations of KCl (13 mM) (Fig. 1), as previously reported for islet β-cells and HIT-T15 cells (9). However, higher KCl (>30 mM) and insulin (100 mM) concentrations were ineffective (data not shown), as described in previous MIN6 cultures (49).

**Regulation of the L-PK Promoter by Glucose and Exogenous Insulin**—In MIN6 cells co-microinjected with a minimal L-PK promoter (base pairs −183 to +10)-firefly luciferase reporter construct (26) and maintained for 6 h at 3 mM glucose, the ratio of firefly to Renilla luciferase activity was in the range 0.02–0.15, with higher values associated with later cell passage numbers. In common with previous studies (26, 52), 30 mM glucose stimulated the L-PK promoter by 3.6 ± 0.07-fold (11).
To determine whether secretion of insulin may be responsible for the increase in L-PK promoter activity at high glucose concentration, we measured the concentrations of insulin present in the incubation medium at the end of the culture period. After 6 h of incubation at 3 and 30 mM glucose, the medium insulin concentrations were 7.08 ± 2.25 and 13.3 ± 4.25 nM, respectively (n = three separate cultures). Thus, elevated glucose stimulated insulin secretion in the cell cultures as expected and over a range to which the L-PK promoter was sensitive (Fig. 2C). We next tested the effect of blocking the exocytosis of insulin in response to glucose with verapamil. In the presence of this inhibitor, 30 mM glucose had no effect on L-PK promoter activity (Fig. 2, A, panels e and f; and B). However, in the presence verapamil, the addition of 20 nM insulin still caused a marked activation of L-PK promoter activity at 3 or 30 mM glucose (Fig. 2, A, panels e–h; and B), demonstrating that the effects of verapamil are unlikely to be due to any nonspecific toxic effects of the drug. Similarly, diazoxide, which opens the ATP-sensitive K⁺ channel (53) and thus prevents cell depolarization and [Ca²⁺], increases (Fig. 3c), also eliminated induction of the L-PK promoter with 30 mM glucose (Fig. 3a). To determine whether the effects of diazoxide or verapamil may be due to an inhibition of Ca²⁺-dependent glucose metabolism (49, 54), we used adenovirally expressed firefly luciferase,² under the control of the strong cytomegalovirus immediate-early gene promoter, to monitor the free cytoplasmic ATP concentration in real time (49). Perifusion of cell populations with 30 mM glucose led to a clear and rapid increase in ATP-dependent luciferase bioluminescence (−15% above basal levels), which was unaffected by the presence of 200 μM diazoxide (Fig. 3d). Finally, inhibition of insulin secretion with the α₂-adrenergic agonist clonidine (1 μM), which has no effect on glucose-induced Ca²⁺, or [ATP] increases (data not shown),³ also strongly inhibited the effect of glucose on the L-PK promoter (Fig. 3h). Clonidine had no impact on the ability of insulin (20 nM) to stimulate the L-PK promoter (Fig. 3h).

To test whether the activation of insulin secretion may be sufficient, in the absence of a nutrient stimulus, to activate the L-PK promoter, we next examined the effects of low concentrations of non-nutrient secretagogues. Increasing the concentration of KCl in the culture medium from 5.4 to 13 mM or adding 10 μM tolbutamide stimulated L-PK promoter activity strongly (Fig. 4, a and c). Measured in perfused cells, each agent caused a clear, but relatively small increase in [Ca²⁺] (Fig. 4, b and d). Importantly, the increase in [Ca²⁺] (Fig. 4, b and d) and insulin secretion (data not shown). However, a clear decrease in cell viability and cell morphology was apparent after 6 h of incubation with these agents (data not shown).

Effect of PI3K Inhibition on the Activation by Glucose of the L-PK Promoter—We next examined the dependence of the effects of glucose on activation of PI3K. The PI3K inhibitor LY294002 completely abolished the effect of 30 mM glucose (Fig. 5a). Indeed, under these conditions, L-PK promoter activity was significantly inhibited at 30 mM glucose compared with 3 mM glucose. To determine whether the inhibitory effect of high glucose may be due to an (undefined) response element lying 3′ to the L-PK glucose response element, we also examined the effects of glucose on the activity of a truncated L-PK promoter bearing only nucleotides −148 to +10. This promoter

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Fig. 1. Regulation of PPI promoter activity by glucose and insulin. MIN6 cells were microinjected with plasmids pPPI-LucFF (0.1 mg/ml) and pCMV-RL (0.05 mg/ml) as described under “Experimental Procedures” before culture for 6 h at the indicated glucose concentrations with the following additions: none (upper panel, panels a and b), 50 μM LY294002 (panels c and d), 20 nM insulin (panels e and f), 13 mM KCl (final concentration; panel g), and 100 μM verapamil (panel h). Normalized PPI promoter activity (obtained from the ratio of firefly to Renilla luciferase activities; pseudo-color) was determined after photon-counting imaging in the presence of luciferin and coelenterazine n. Shown are typical regions (~0.06 mm²) from a total injected area of ~0.25 mm². Scale bar = 50 μM. The pseudo-color bar represents the ratio of firefly luciferase (reporting PPI promoter activity) to Renilla luciferase activity (CMV promoter). Combined data (means ± S.E.) from three separate experiments are shown in the lower panel and report observations on the total number of individual cells given on the bars. ***p < 0.001 for the effect of glucose, insulin, or KCl.

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separate experiments, n = 108 and 126 cells incubated at 3 and 30 mM glucose, respectively; p < 0.001 for the effect of 30 mM glucose) (Fig. 2, A, panel b; and B). This effect was mimicked by the addition of exogenous insulin (20 nM). Thus, in 11 experiments on separate cultures, 20 nM insulin increased the activity of the L-PK promoter in cells maintained at 3 mM glucose by 3.3 ± 0.002-fold (n = 108 control and 67 insulin-stimulated cells; p < 0.001) (Fig. 2, A, panel c; and B). By contrast, insulin was entirely without effect at stimulatory glucose concentrations (30 mM) (Fig. 2, A, panels b and d; and B). At 3 mM glucose, the effect of insulin was clearly apparent at low physiological concentrations of the hormone, with 1 nM insulin providing >50% maximal stimulation of promoter activity (Fig. 2C). By contrast, 100 nM insulin failed to stimulate L-PK promoter activity significantly (Fig. 2C).
was also significantly inhibited by 30 versus 3 mM glucose (Fig. 5a). Confirming that the effects of LY294002 were likely to be specific for PI3K, overexpression of a dominant-negative form of the PI3K adaptor subunit (Δp85) lacking the domain responsible for binding the catalytic subunit of PI3K (p110) (38, 41) led to activation of the L-PK promoter in cells maintained at 3
mM glucose, but a powerful inhibition of promoter activity in cells maintained at 30 mM glucose (Fig. 5).

To determine whether the inositol phospholipid or protein kinase activities of PI3K (55) mediated the effects of glucose and insulin on L-PK promoter activity, we examined the effects of overexpressing the inositol-3-phosphate phosphatase PTEN (phosphatase and tensin homologue) (56). PTEN expression completely ablated the activation of the L-PK promoter by glucose (Fig. 5c).

Effects of Glucose and Insulin on Glucokinase Promoter Activity—Adenovirally mediated overexpression of liver glucokinase (57, 58) strongly increased L-PK promoter activity both at 3 mM glucose (from a firefly/R. reniformis luminescence ratio of 0.008 (n = 12 null virus-infected cells) to 0.196 (n = 16 GK virus-infected cells)) and at 30 mM glucose (from 0.129 (n = 39 null cells) to 0.241 (n = 9 infected cells)). However, at 3 mM glucose, insulin (20 nM) still elicited a further induction of L-PK promoter activity after the overexpression of GK (firefly/R. reniformis luminescence ratio of 0.73 ± 0.08, n = six insulin-treated cells). Furthermore, neither 30 mM glucose nor exogenous insulin (20 mM) affected the activity of a reporter construct encoding 4 kilobase pairs of the islet-specific glucokinase promoter (Fig. 6a) (59). Finally, immunocytochemical analysis revealed no detectable change in the level of glucokinase protein after 6 h culture at 3 or 30 mM glucose or at 3 mM glucose plus insulin (data not shown).

Effect of Glucose and Insulin on Intracellular Free ATP Concentration—To examine further whether the effects of insulin or elevated glucose may be due to an enhancement of glucose metabolism independent of glucokinase gene expression, we used adenovirally expressed firefly luciferase as described above (49). Demonstrating that this assay reported increases in glycolytic flux, overexpression of glucokinase with the adenoviral vector dramatically altered the responses of intracellular [ATP] to changes in glucose concentration (Fig. 6b). In control (null virus-infected) cells, increases in apparent cellular free [ATP] were not evident until the glucose concentration was raised to 30 mM (Fig. 6b, open circles) and were substantially blocked by the GK inhibitor mannoheptulose (data not shown). By contrast, in GK-overexpressing cells, intracellular free [ATP] was increased considerably by 3 mM glucose, but lowered when glucose was further raised to 30 mM (Fig. 6b, closed circles). This drop presumably reflects ATP consumption by the glucokinase reaction itself under conditions in which distal steps in glycolysis or glucose oxidation exert a significant control strength (58). The increase in luminescence of 15–20% seen in control cells in response to 30 mM glucose (Figs. 3, d; and 6, b–d) was unaffected by culture in the presence of insulin for 6 h (Fig. 6c) or after acute (5 min) exposure to the hormone (Fig. 6d). These experiments also reveal that the contribution of changes in [ATP] to the changes in the apparent promoter activity reported with firefly luciferase constructs are unlikely to exceed 20% (and have therefore been ignored).

Role of PI3K in the Effects of AMPK-α2—We have previously demonstrated that inhibition of the α2 form of AMPK activates the L-PK promoter, mimicking the effect of glucose (52). To determine whether AMPK lies upstream or downstream of PI3K, we examined the effect of PI3K inhibition when AMPK activity had been inhibited by microinjection of anti-AMPK-α2 antibodies (52) (Fig. 7). The stimulatory effect of inhibiting
AMPK-α2 activity was also strongly reversed by inhibition of PI3K with LY294002 (Fig. 7).

**DISCUSSION**

**Role of Insulin Secretion in the Regulation of the PPI Gene by Glucose**—This study demonstrates that, in the highly glucose-responsive MIN6 β-cell line, stimulation of exocytosis by glucose plays a key role in activating the L-PK gene as well as PPI gene expression. It should be stressed that this study was performed under conditions different from those of our recent report (14), in which glucose was able to activate the PPI promoter in MIN6 cells relatively weakly (1.5-fold) and independently of insulin secretion. Although not investigated in the present study, it seems likely that changes in the distribution (38, 40) or DNA-binding activity (60) of the homeodomain A3-binding protein PDX-1 (pancreatic duodenum homeobox-1; formerly termed IPF-1, IDX-1, STF-1, and IUF-1) (61) are likely to play an important role in this activation.

**Comparison of the Role of Insulin Secretion in the Regulation of PPI and L-PK Genes by Glucose**—In this report, we demonstrate that the L-PK gene, in common with the PPI gene, can be regulated largely by glucose through the stimulation of insulin secretion. Thus, we have used three approaches to distinguish between the roles of 1) insulin secretion, 2) changes in [Ca^{2+}], and 3) glucose metabolism in the regulation of transcription. It should be emphasized that our measurements of [Ca^{2+}], and intracellular free [ATP] (Figs. 3, 4, and 6) were made only over the short term (minutes) compared with the longer incubations (hours) used in measurements of gene expression. These should therefore only be taken as a guide to the changes in these parameters during the full time course of measurements of gene expression (6 h). Nevertheless, the former measurements were considered important since several maneuvers that block secretion (e.g. by preventing increases in [Ca^{2+}]), and intracellular free [ATP]) (Figs. 3, 4, and 6) were made only over the short term (minutes) compared with the longer incubations (hours) used in measurements of gene expression. These should therefore only be taken as a guide to the changes in these parameters during the full time course of measurements of gene expression (6 h). Nevertheless, the former measurements were considered important since several maneuvers that block secretion (e.g. by preventing increases in [Ca^{2+}]), and intracellular free [ATP]) (Figs. 3, 4, and 6) were made only over the short term (minutes) compared with the longer incubations (hours) used in measurements of gene expression. These should therefore only be taken as a guide to the changes in these parameters during the full time course of measurements of gene expression (6 h). Nevertheless, the former measurements were considered important since several maneuvers that block secretion (e.g. by preventing increases in [Ca^{2+}]), and intracellular free [ATP]).

**Fig. 5.** Effect of inhibiting PI3K function on L-PK promoter activity. a, MIN6 cells were microinjected as described under "Experimental Procedures" with plasmids pCMV-RL and pLPK-Luc<sub>pp</sub> or p(-150)LPK-Luc<sub>pp</sub> (-150L-PK) (as indicated) before 6 h of culture at 3 or 30 mM glucose as indicated in the absence or presence of 50 μM LY294002. *** and *, p < 0.001 and p < 0.05 for the effect of glucose, respectively. b, same as a, but with co-microinjection of the plasmid encoding Ap85 or the corresponding empty vector (pSRa; Control). ***, and +++, p < 0.001 for the effect of 30 μM glucose and LY294002, respectively. c, same as a, but after co-injection of the expression plasmid encoding PTEN or the empty vector (pCMV5; Control) as indicated. +++, p < 0.01 for the effect of PTEN expression relative to the control (30 mM glucose).

**Fig. 6.** Impact of insulin and glucose on glucokinase promoter activity and glucose metabolism in single cells. a, cells were microinjected with plasmids pGK4-Luc (0.1 mg/ml) and pRL-CMV (0.05 mg/ml) and incubated for 6 h at the indicated glucose concentrations or at 3 mM glucose in the presence of 20 nM insulin (Ins). b–d, shown is the response of cytoplasmic [ATP] in MIN6 cells to 30 mM glucose. b, cells were transduced with adenoviral luciferase (AdCMVluc; see "Experimental Procedures" with adenoviruses expressing GK (closed circles; GK<sup>+</sup>) or empty virus (open circles). 16 h after infection, the cells were preincubated for 1 h in KRB buffer at 0 mM glucose before perfusion in the presence of 5 μM luciferin for detection of luciferase light emission (see "Experimental Procedures") at the indicated glucose concentrations. c, cells expressing AdCMVluc alone were incubated for 6 h in culture medium containing 3 mM glucose and either 20 mM insulin (closed circles) or no insulin (open circles), and the response of [ATP] to a change in glucose from 3 to 30 mM glucose was measured as shown. d, cells were incubated for 16 h in medium containing 3 mM glucose and then subsequently perfused with KRB buffer supplemented with 3 mM glucose alone (open circles) or 3 mM glucose and 20 mM insulin (closed circles). After 5 min of pre-perfusion, the glucose concentration was then changed to 30 mM as shown (≥20 mM insulin as appropriate). The time bars represent 100 s.
potent inhibitor of L-PK promoter induction (Fig. 3b). Together, these data suggest that insulin secretion, but not an increase in glucose metabolism or increases in $[\text{Ca}^{2+}]_{i}$, is the chief trigger for L-PK gene activation by glucose in the MIN6 $\beta$-cell model.

The induction of the PPI promoter by insulin reported here is consistent with recent reports (9) and with the effects of a range of nutrient secretagogues in HIT-T15 cells (15) and MIN6 cells (60). Strikingly, higher concentrations of either KCl or tolbutamide exerted no stimulatory effect on the apparent transcriptional activity of either the PPI or L-PK gene or were toxic effects on cell function. These may become particularly pronounced over longer periods of culture and may remain to be elucidated.

Role of AMPK—AMPK is a critical event in the regulation of L-PK promoter activity by glucose (52). Activation of AMPK with 5-aminoimidazole-4-carboxamide ribonucleoside, which is converted into the AMP analogue 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate (67), leads to the suppression of L-PK gene expression in liver (30, 68) as well as in $\beta$-cell-derived HIT-T15, INS-1 (69), and MIN6 (52) cells. Specific inhibition of the $\alpha$2 isoform of AMPK by antibody microinjection fully mimics the effect of high glucose concentrations on L-PK promoter activity in MIN6 cells (52). Incubation of islets with 5-amino-4-imidazolecarboxamide riboside leads to a weak activation of insulin secretion at low glucose concentrations, but then substantially inhibits further increases in insulin release as the glucose concentration is elevated (69, 70). It is therefore possible, but at present not proven, that inhibition of AMPK activity, achieved by the microinjection of antibodies into single cells, may lead to an activation of insulin secretion at low glucose concentrations. The released insulin may then, in turn, bind to insulin receptors and activate PPI and L-PK gene transcription.

Consistent with this view, the activation of L-PK promoter activity resulting from the microinjection of anti-AMPK-α2 antibodies was also sensitive to PI3K blockade (Fig. 7). These observations suggest that AMPK lies either on a signaling pathway upstream of PI3K or on a separate signaling pathway that is responsible for repression of the L-PK gene in low glucose.

This study supports the view that the L-PK promoter may be regulated by insulin through both glucose metabolism-dependent and -independent pathways. The relative importance of these pathways may, however, depend on the cell type and culture conditions. First, as demonstrated in this study on MIN6 cells, insulin can act via a signaling pathway that involves PI3K and presumably a subsequent protein kinase cascade, perhaps involving phospholipid-dependent kinase-1 and protein kinase B/Akt (71). Second, in liver and INS-1 $\beta$-cells, changes in the intracellular concentration of a glucose metabolite such as Glu-6-P may also activate L-PK gene transcription directly. Thus, 2-deoxyglucose increased the level of L-PK mRNA in INS-1 cells ($\sim 1.5$-fold increase at $10 \text{ mM}$ 2-deoxyglucose) (3), albeit more weakly than glucose (3-fold increase at 10 mM). The poorer efficacy of 2-deoxyglucose compared with glucose in L-PK gene transcription may reflect its inability to
stimulate insulin secretion efficiently (72). Furthermore, inhibition of PI3K activity and dephosphorylation of 3′-phosphoinositides both blocked the effects of glucose, implicating insulin receptor activation as an early event in the signal transduction pathway leading to transcriptional activation. Importantly, these data also suggest that the PPI and L-PK genes are regulated directly by insulin, and not indirectly as a result of induction of GK and stimulation of glucose phosphorylation. Thus, GK gene expression (Fig. 6a) and glucose metabolism (Fig. 6, b and c) were unaltered by culture with insulin in this model, consistent with the absence of changes in islet GK gene expression in starved versus fed animals (32). It therefore seems likely that the increase in L-PK promoter activity (this work) and L-PK mRNA levels (73) may be due to the activation of insulin secretion under these conditions (73).

**Potential Signaling Pathways and Transcription Factors Involved in the Regulation of L-PK Gene Transcription by Glucose and Insulin**—An important question is to define the molecular target of the two potential signaling mechanisms leading to the induction of the L-PK gene. One intriguing possibility is that both pathways converge on a single target molecule, which may be phosphorylated by an insulin-activated protein kinase. Changes in the intracellular levels of Glu-6-P/xyulose 5-phosphate could then interact with this pathway by influencing the phosphorylation state of this target through changes in the activity of a protein phosphatase (such protein phosphatase 2A) (30, 34).

What may be the target of the putative phosphorylation event described above? Both SREBP-1c (74) and USF1/USF2 (26, 27) have been implicated in the regulation by glucose of the L-PK promoter in liver, but their respective roles are at present uncertain (75). Each of these factors binds to oligonucleotides corresponding to the L-PK L4 box, with USF1/2 binding with the greatest avidity (33). In islet β-cells, injection of blocking antibodies demonstrated that USF2 function is essential for L-PK induction by glucose (26). However, a dominant-negative form of USF2 was found to be ineffective in inhibiting L-PK gene induction in INS-1 cells (28) and liver cells (29). We have found that suppression of SREBP-1c function in MIN6 cells with a dominant-negative form of the protein (74) markedly reduces glucose-activation of the promoter, suggesting that this factor is required for the regulation of the gene in β-cells. However, the L-PK promoter appears to be less dependent upon SREBP-1c activity than other glucose-regulated genes in β-cells and liver cells (74–76).

**Conclusion**—These studies demonstrate that both the PPI and L-PK promoters can be regulated by insulin in MIN6 β-cells. In this model system, release of insulin and the activation of a PI3K-dependent signaling pathway may be the predominant mechanisms by which glucose activates glucose-sensitive promoters (Fig. 8).

**Acknowledgments**—We thank Alan Leard and Dr. Mark Jepson (Bristol Medical Research Council Imaging Facility) for assistance with imaging experiments. We thank Dr. C. Zhao for measurements of insulin secretion, Drs. L. Agius and C. Newgard for providing GK adenovirus, and Drs. Isabelle Leclerc and Axel Kahn for useful discussions.

**REFERENCES**

1. Welsh, M., Nielsen, D. A., MacKrell, A. J., and Steiner, D. F. (1985) *J. Biol. Chem.* 260, 13590–13594
2. Docherty, K., and Clark, A. E. (1994) *FASEB J.* 8, 20–27
3. Marie, S., Diaz-Guerra, M.-J., Miquerol, L., Kahn, A., and Iynedjian, P. B. (1993) *J. Biol. Chem.* 268, 23881–23890
4. Bruun, T., Roche, E., Kim, K. H., and Prentki, M. (1994) *J. Biol. Chem.* 269, 18095–18101
5. Yasuda, K., Yamada, Y., Inagaki, N., Yano, H., Okamoto, Y., Tsuji, K., Fukumoto, H., Imura, H., Seino, S., and Seino, Y. (1992) *Diabetes* 41, 76–81
6. Welsh, G. C., Akbar, M. S., Zhao, C. J., and Steiner, D. F. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 5773–5778
7. Vaulont, S., and Kahn, A. (1994) *FASEB J.* 8, 20–27
8. Rutter, G. A., Tavare, J. M., and Palmer, D. G. (2000) *News Physiol. Sci.* 15, 149–154
9. Leibiger, I. B., Leibiger, B., Moede, T., and Berggren, P. O. (1998) *Mol. Cell 1*, 953–958
10. Rutter, G. A. (1999) *Curr. Biol.* 9, R443–R445
11. Effrat, S., Surana, M., and Fleischer, N. (1991) *J. Biol. Chem.* 266, 11141–11143
12. Gofan, M. S., Moss, L. G., and Rutter, W. J. (1990) *J. Biol. Chem.* 265, 22063–22066
13. de Vargas, L. M., Sobolewski, J., Siegel, R., and Moss, L. G. (1997) *J. Biol. Chem.* 272, 26573–26577
14. Kennedy, H. J., Rafiq, I., Pouli, A. E., and Rutter, G. A. (1999) *Biochem. J.* 342, 275–280
15. Goodman, S., Kenna, S., and Ashcroft, S. J. H. (1992) *Biochem. J.* 285, 563–568
16. Kalkani, R. N., Bruning, J. C., Winnay, J. N., Postic, C., Magnuson, M. A., and Kahn, C. R. (1999) *Cell 96*, 329–339
17. Prentki, M., Thorner, K., and Corkey, B. E. (1997) *Diabetologia* 40, S32–S41
18. Vaulont, S., Munnich, A., Decaux, J. F., and Kahn, A. (1996) *J. Biol. Chem.* 271, 7623–7625
19. Cui, M. H., Porteu, A., Kahn, A., and Vaulont, S. (1993) *J. Biol. Chem.* 268, 13769–13772
20. Towle, H. C. (1995) *J. Biol. Chem.* 270, 23235–23238
21. Shih, H., and Towle, H. C. (1994) *J. Biol. Chem.* 269, 9380–9387
22. Luo, X. C., and Kim, K. H. (1990) *Nucleic Acids Res.* 18, 3249–3254
23. Lopez, J. M., Bennett, M. K., Sanchez, H. B., Rosenfeld, J. M., and Osborne, T. E. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 1049–1053
24. Su, H. S., Latasa, M. J., Moon, Y., and Kim, K. H. (2000) *J. Nutr.* 130, 3128–3133
25. Doiran, B., Cui, M.-H., Chen, R., and Kahn, A. (1996) *J. Biol. Chem.* 271, 5321–5324
26. Kennedy, H. J., Viollet, B., Rafiq, I., Kahn, A., and Rutter, G. A. (1997) *J. Biol. Chem.* 272, 20636–20641
27. Le Francois-Martinez, A. M., Martinez, A., Antoine, B., Raymondjean, M., and Kahn, A. (1995) *J. Biol. Chem.* 270, 2640–2643

* C. J. Zhao, C. Andreolas, and G. A. Rutter, manuscript in preparation.
* G. da Silva Xavier, C. Andreolas, and G. A. Rutter, unpublished data.
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28. Qian, J., Kaytor, E. N., Towe, H. C., and Olson, L. K. (1999) Biochem. J. 341, 315–322
29. Kaytor, E. N., Shih, H., and Towe, H. C. (1997) J. Biol. Chem. 272, 7525–7531
30. Foretz, M., Carling, D., Guichard, C., Ferre, P., and Foufelle, F. (1998) J. Biol. Chem. 273, 14767–14771
31. Yamada, K., and Noguchi, T. (1999) Biochem. J. 337, 1–11
32. Iynedjian, P. B., Pilot, P.-R., Nouspikel, T., Milburn, J. L., Quaade, C., Hughes, S., Ueda, C., and Newgard, C. B. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 7838–7842
33. Doiron, B., Cuif, M. H., Kahn, A., and Diaz-Guerra, M. J. M. (1994) J. Biol. Chem. 269, 10213–10216
34. Kahn, A. (1997) Biochimie 79, 113–118
35. Cuif, M. H., Doiron, B., and Kahn, A. (1997) FEBS Lett. 417, 81–84
36. Tiedge, M., Steffelk, H., Elsner, M., and Lenzen, S. (1999) Diabetes 48, 514–523
37. Liang, Y., Najafi, H., and Matschinsky, F. M. (1990) J. Biol. Chem. 265, 16863–16866
38. Rafiq, I., da Silva Xavier, G., Hooper, S., and Rutter, G. A. (2000) J. Biol. Chem. 275, 15977–15984
39. Miyazaki, J., Araki, K., Yamato, E., Ikegami, H., Asano, T., Shibasaki, Y., Oka, Y., and Yamamura, K. (1990) Endocrinology 127, 126–132
40. Rafiq, I., Kennedy, H. J., and Rutter, G. A. (1998) J. Biol. Chem. 273, 23241–23247
41. Dhand, R., Haru, K., Hiles, I., Bax, B., Gout, I., Panayotou, G., Fry, M. J., Yonezawa, K., Kasuga, M., and Waterfield, M. D. (1994) EMBO J. 13, 511–521
42. Thornton, C., Snowden, M. A., and Carling, D. (1998) J. Biol. Chem. 273, 12443–12450
43. Alcrosow, E., Zhao, C., and Rutter, G. A. (2000) Diabetes 49, 1149–1155
44. Rutter, G. A., Burnett, P., Rizzuto, R., Brini, M., Murgia, M., Pozzan, T., Tavare, J. M., and Denton, R. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5489–5494
45. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
46. Rutter, G. A., Kennedy, H. J., Wood, C. D., White, R. H. R., and Tavare, J. M. (1998) Biochem. J. 352, R285–R290
47. Rutter, G. A., Theler, J.-M., Murta, M., Wollheim, C. B., Pozzan, T., and Rizzuto, R. (1990) J. Biol. Chem. 265, 22385–22390
48. Emmanouilidou, E., Teschemacher, A., Pouli, A. E., Nicholls, L. I., Seward, E. P., and Rutter, G. A. (1999) Curr. Biol. 9, 915–918
49. Kennedy, H. J., Pouli, A. E., Jouaville, L. S., Rizzuto, R., and Rutter, G. A. (1999) J. Biol. Chem. 274, 13281–13289
50. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Kintzler, K. W., and Vogelstein, B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2509–2514
51. Herman, M., Ashcroft, S., Docherty, K., Edlund, H., Edlund, T., Goodson, S., Imura, H., Kennedy, G., Madsen, O., Meloul, D., Moss, L., Olson, K., Permutt, M. A., Philippe, J., Robertson, R. P., Rutter, W. J., Serup, P., Stein, R., Steiner, D., Tsai, M. J., and Walker, M. D. (1995) Diabetes 44, 1092–1094
52. da Silva Xavier, G., Leclerc, I., Salt, I. P., Doirion, B., Hardie, D. G., Kahn, A., and Rutter, G. A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4023–4028
53. Bryan, J., and Aguilar-Bryan, L. (1997) Curr. Opin. Cell Biol. 9, 553–559
54. Gilson, P., and Henguin, J. C. (1992) J. Biol. Chem. 267, 20713–20720
55. Bondeva, T., Pirsa, L., Bulgarelli-Leva, G., Rubio, I., Wetzker, R., and Wynnman, M. P. (1998) Science 282, 293–296
56. Maehama, T., and Dixon, J. E. (1998) J. Biol. Chem. 273, 13375–13378
57. Beckers, C., Noel, R. J., Johnson, J. H., Lynch, R. M., Hirose, H., Tokuyama, Y., Bell, G. I., and Newgard, C. B. (1996) J. Biol. Chem. 271, 390–394
58. Berman, H. K., and Newgard, C. B. (1998) Biochemistry 37, 4543–4552
59. Van der Brugge, J. P. (1993) Biochem. J. 293, 1–13
60. Wu, H., MacFarlane, W. M., Tadayyon, M., Arch, J. R., James, R. F., and Docherty, K. (1999) Biochem. J. 344, 813–818
61. Ohlsson, H., Karlsson, K., and Edlund, T. (1995) EMBO J. 14, 4251–4259
62. ApSimon, C. A., Qian, W. J., Roger, M. G., Kulkarni, R. N., Kahn, C. R., and Kennedy, R. T. (2000) J. Biol. Chem. 275, 22331–22338
63. Pauli-Magnus, C., von Richert, O., Burk, O., Ziegler, A., Mettang, T., Eichelaub, M., and Fromm, M. F. (2000) J. Pharmacol. Exp. Ther. 293, 376–382
64. MacFarlane, W. M., Smith, S. B., James, R. F. L., Clifton, A. D., Doza, Y. N., Cohen, P., and Docherty, K. (1997) J. Biol. Chem. 272, 20936–20944
65. Buteau, J., Roduit, R., Susini, S., and Prentki, M. (1999) Diabetologia 42, 586–584
66. Harvey, J., McKay, N. G., Walker, K. S., Van der, K., Kaay, J. K. J., Downes, C. P., and Ashford, M. L. J. (2000) J. Biol. Chem. 275, 4660–4669
67. Hardie, D. G., Carling, D., and Carlson, M. (1998) Annu. Rev. Biochem. 67, 821–854
68. Leclerc, I., Kahn, A., and Doirion, B. (1998) FEBS Lett. 431, 180–184
69. Salt, I. P., Johnson, G., Ashcroft, S. J., and Hardie, D. G. (1998) Biochem. J. 335, 533–539
70. Zhang, S., and Kim, K. H. (1995) J. Endocrinol. 147, 33–41
71. Kandel, E. S., and Hay, N. (1999) Exp. Cell Res. 253, 210–229
72. Kilo, C., Devrim, S., Bailey, R., and Recant, L. (1967) Diabetes 16, 377–385
73. Wang, H., and Inyedjian, P. B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4372–4377
74. Foretz, M., Pacot, C., Hogrel, J. L., Marchand, P., Guichard, C., Leclercq, P., Berthelier-Lubrano, C., Spiegelman, B. M., Kim, J., Ferre, P., and Foufelle, F. (1999) Mol. Cell. Biol. 19, 3760–3768
75. Morizumi, S., Gourdon, L., Lefrancois-Martinez, A. M., Kahn, A., and Raymond-jean, M. (1998) Gene Expr. 7, 103–113
76. Mater, R. M., Thelen, A. P., Fan, D. A., and Jump, D. B. (1999) J. Biol. Chem. 274, 32725–32732