

Glucose Modulation of Insulin mRNA Levels Is Dependent on Transcription Factor PDX-1 and Occurs Independently of Changes in Intracellular Ca²⁺

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Glucose regulates insulin production in pancreatic β -cells in the long term by stimulating insulin gene transcription. These effects are partially mediated through the activity of a homeodomain transcription factor, PDX-1, which binds to four sites within the human insulin gene promoter. The availability of a human β -like cell line, NES2Y, which lacks PDX-1 but expresses the insulin gene, allowed us to determine whether PDX-1 was essential for the stimulatory effect of glucose on insulin mRNA levels. In NES2Y cells, glucose had no effect on the insulin gene promoter linked to a firefly luciferase reporter or on endogenous insulin mRNA levels. However, in NES2Y cells stably transfected with PDX-1 (NES-PDX-1), glucose exhibited a marked stimulatory effect on both the insulin promoter (5 ± 0.2 -fold, $n = 6$) and insulin mRNA levels (4.8 ± 0.5 -fold, $n = 4$). NES2Y cells were derived from a patient with persistent hyperinsulinemic hypoglycemia of infancy; the cells therefore lacked operational ATP-sensitive potassium channels, which results in the failure to control depolarization-dependent intracellular Ca²⁺ signaling. Despite the loss of control of Ca²⁺ channel activity, NES-PDX-1 cells maintained normal glucose-responsive insulin gene regulation. These results demonstrate that glucose modulation of insulin mRNA levels is dependent on the activity of PDX-1 and that these effects are independent of changes in intracellular Ca²⁺ concentrations. *Diabetes* 49:418–423, 2000

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[Ca²⁺]_i, intracellular Ca²⁺ concentration; EMSA, electrophoretic mobility shift assay; IAAP, islet amyloid polypeptide; K_{ATP}, ATP-sensitive potassium channels; NES-PDX-1, NES2Y stably transfected with PDX-1; PHHI, persistent hyperinsulinemic hypoglycemia of infancy; SAPK2, stress-activated protein kinase 2.

Glucose stimulates insulin secretion from pancreatic β -cells through its ability to affect closure of plasma membrane ATP-sensitive potassium channels (K_{ATP}). The resultant membrane depolarization activates voltage-sensitive Ca²⁺ channels and consequently leads to influx and Ca²⁺-stimulated exocytosis of insulin. Glucose also stimulates translation of preformed insulin mRNA (within minutes) and increases the levels of insulin mRNA. This latter effect occurs through a combination of increased stability of insulin mRNA and increased transcription of the insulin gene (1).

Transcription of the insulin gene is regulated by promoter sequences located within the region up to 350 bp from the start site (2). Mutagenesis within this region has failed to identify a discrete glucose-responsive element (3), as is the case for other metabolically regulated genes, such as S14 and those encoding L-type pyruvate kinase (4). This finding suggests that multiple cis-acting elements within the insulin promoter that interact synergistically may be involved in mediating the stimulatory effect of glucose (3). In keeping with this model, it is noticeable that the DNA-binding activity of a number of β -cell transcription factors that recognize discrete elements within this promoter region is stimulated by glucose. These include the homeodomain transcription factor PDX-1 (5–7), an uncharacterized factor, RIPE3b1, which binds to the C1 element of the rat insulin II gene promoter (8), and a factor that binds to a negative regulatory element of the human insulin promoter (9).

We have recently shown that glucose activates PDX-1 through a cell-signaling pathway involving phosphoinositide 3-kinase and stress-activated protein kinase 2 (SAPK2/p38) (10). Stimulation of the pathway leads to phosphorylation and activation (DNA-binding) of a cytoplasmic form of PDX-1 that translocates to the nucleus (11). In addition to insulin, PDX-1 binds to the promoter of a number of genes that are expressed preferentially in the β -cell, including those encoding GLUT2 (12), glucokinase (13), and islet amyloid polypeptide (IAAP) (14). The exact role of PDX-1 in regulating these genes is unclear. It is not essential for insulin gene expression, because high levels of insulin mRNA have been observed in β -cells in which the levels of PDX-1 have been reduced by

using an antisense RNA strategy (15). Also, in the human NES2Y β -cell line, high levels of insulin mRNA are observed in the absence of functional PDX-1 (16).

NES2Y cells are a proliferating human β -cell line derived from a patient with persistent hyperinsulinemic hypoglycemia of infancy (PHHI) (17). Previous studies have established that these cells lack functional levels of the PDX-1 protein, making them a unique tool to evaluate the role of PDX-1 in the glucose regulation of insulin mRNA production (16). In addition to defective expression of PDX-1, NES2Y cells lack functional K_{ATP} channels as in the tissue of origin. As a consequence, NES2Y cells demonstrate a loss of normal Ca^{2+} signaling, with a resultant loss of stimulus-secretion coupling of insulin release (18). Ca^{2+} influx into pancreatic β -cells represents the critical step in the regulation of insulin secretion (18). However, the role of Ca^{2+} in insulin mRNA production remains unclear. In the present study, NES2Y cells lacking both PDX-1 and normal voltage-gated Ca^{2+} channel activity were used to establish that PDX-1 is absolutely required for the glucose regulation of insulin mRNA production and that these events are not dependent on changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

RESEARCH DESIGN AND METHODS

Cell culture. NES2Y cells were derived from islets of Langerhans isolated from the pancreas of a patient with PHHI, as previously described (16). Isolated human islets of Langerhans were prepared as previously described (17). MIN6 cells (20) were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma), 5 mmol/l D-glucose, and 2 mmol/l L-glutamine. All experiments were performed with MIN6 cells between passage 27 and 33.

Plasmids. Reporter constructs pGL-LUC and pGL-LUC200 and the pCR3-PDX-1 construct were used as described previously (11). Plasmid DNA was prepared using the endotoxin-free maxiprep method (Qiagen, Hilden, Germany) and was quantitated spectrophotometrically.

Transfections. Transfections and selection of G418-resistant NES2Y-PDX-1 cells were performed as previously described (11).

Luciferase assays. Luciferase assays were performed as previously described (11).

Electrophysiology. All data were obtained from control and NES2Y cells stably transfected with PDX-1 (NES-PDX-1) cells using the standard whole-cells-recording configuration of the patch-clamp technique as previously described (21).

Measurement of cytosolic Ca^{2+} by Fura-2 microfluorometry. For experimental purposes, NES2Y and NES-PDX-1 cells were maintained for up to 58 h on poly-D-lysine (50 μ g/ml)-treated coverslips in standard RPMI 1640 media supplemented with 10% fetal calf serum (Gibco, Paisley, U.K.). All cells were loaded with 0.5 μ mol/l Fura-2-AM (Sigma) in 1 ml RPMI 1640 medium for 20–30 min at 37°C before experimentation. The composition of the basic perfusion medium was as follows: 137 mmol/l NaCl, 5.36 mmol/l KCl, 0.81 mmol/l $MgSO_4$, 0.34 mmol/l Na_2HPO_4 , 0.44 mmol/l KH_2PO_4 , 4.17 mmol/l $NaHCO_3$, 10 mmol/l HEPES, and 1.26 mmol/l $CaCl_2$. This medium was gassed with air for 20 min, glucose was added to a final concentration of 2.02 mmol/l, and pH was set to 7.4 with NaOH. In instances in which the perfusion medium contained 40 mmol/l KCl, the concentration of NaCl was adjusted accordingly. To quantify changes in the $[Ca^{2+}]_i$ (20) as the difference between basal $[Ca^{2+}]_i$ and the peak rise in $[Ca^{2+}]_i$, an *in vitro* calibration procedure was used as previously described (17). Results were tested for significance by using a Student's unpaired t test. P values <0.05 were considered significant.

Northern blotting. Total RNA was prepared by use of the RNeasy system (Qiagen) according to the manufacturer's protocols. Northern blot analysis was performed as previously described (16). Phosphorimaging analysis was performed by using the Bio-Rad Molecular Imager System (Richmond, CA) on a G2-252 Scanner, and the values were quantified by using the Bio-Rad Molecular System Analyst program.

Electrophoretic mobility shift assays. Nuclear extracts were prepared, and electrophoretic mobility shift assays (EMSAs) were performed as previously described (22).

Western blotting. Nuclear extract samples of 1 μ g were fractionated by SDS-PAGE and blotted as previously described (16). Anti-PDX-1 antibody was kindly provided by Dr. C.V. Wright (Vanderbilt University, Nashville, TN).

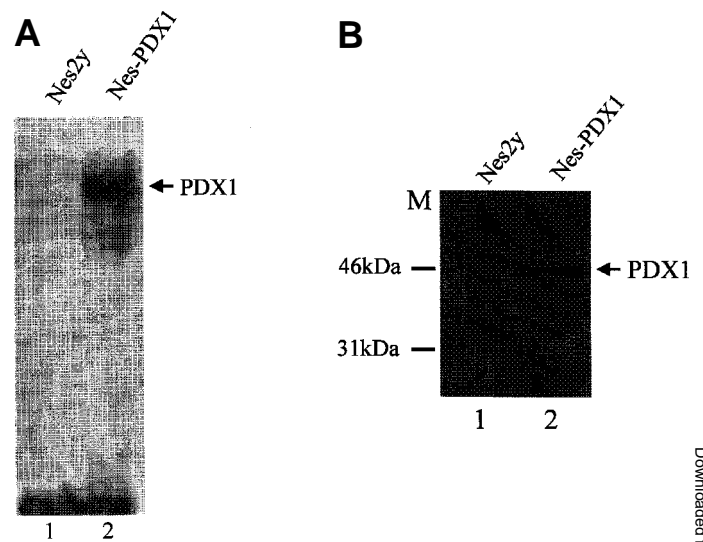


FIG. 1. A: EMSA analysis of PDX-1 binding activity in NES2Y and NES-PDX-1 cell nuclear extracts, using the A3 site of the human insulin gene promoter as a probe. PDX-1 DNA-binding activity is highlighted by the arrow. B: Western blot analysis of PDX-1 in NES2Y and NES-PDX-1 cells. Nuclear extracts from the indicated cell lines were analyzed for the PDX-1 protein using a specific anti-PDX-1 antibody. PDX-1, indicated by the arrow, has a molecular size of 46 kDa. All samples were prepared from cells stimulated in 20 mmol/l glucose and equalized for protein concentration.

RESULTS

NES2Y is a proliferating human β -cell line derived from a patient with PHHI (17). Previous studies have established that in addition to defective control of insulin secretion, these cells also lack functional levels of the homeodomain transcription factor PDX-1 (16). Analyses of the binding activities of other characterized insulin gene regulatory proteins, such as upstream stimulatory factor, which binds to the E2 site, and insulin enhancer factor-1, which binds to the E1 site, have shown that these factors are unaffected in NES2Y cells (16). The less well-characterized factor RIPE3b1 also appears to be normal and unaffected in the NES2Y cells. Our previous studies have shown that transient overexpression of the PDX-1 protein can restore normal glucose regulation of the human insulin gene promoter in these cells (16). Stable overexpression of PDX-1 in the present study resulted in the formation of the NES-PDX-1 cell line. To confirm overexpression of PDX-1 in these cells, nuclear extracts prepared from these and untransfected NES2Y cells were analyzed by an EMSA (Fig. 1A) by using the A3 site (22) of the human insulin gene promoter as the probe. PDX-1 binding to the A3 site was observed in the NES-PDX-1 cells, whereas the untransfected NES2Y cells had no detectable PDX-1 DNA-binding activity (Fig. 1A). Specificity of the EMSA was confirmed by demonstrating competition with oligonucleotide Bm1, which contains a mutation within the A3 sequence that does not affect PDX-1 binding, whereas oligonucleotide Bm2, which contains a mutation within the A3 sequence that abolishes PDX-1 binding, did not compete (22). In addition, an anti-PDX-1 antibody competed for binding of the expressed protein to oligonucleotide B. Western blot analysis of the same nuclear extracts confirmed the lack of PDX-1 in NES2Y cells, whereas PDX-1 (46 kDa) was clearly visible in the NES-PDX-1 cells (Fig. 1B).

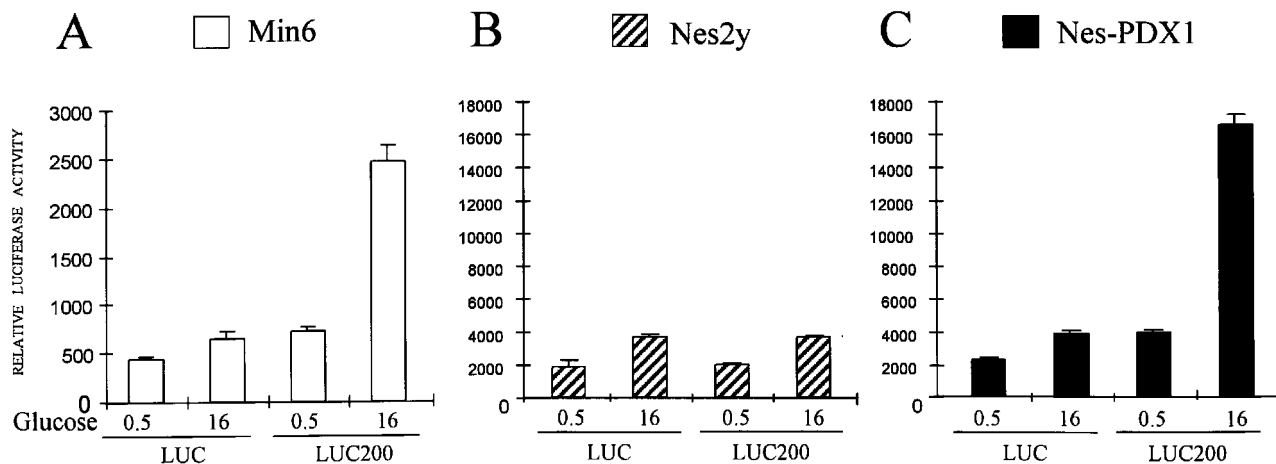


FIG. 2. Insulin promoter activity in Min6, NES2Y, and NES-PDX-1 cell lines. Min6 cells (A), NES2Y cells (B), and NES-PDX-1 cells (C) were transfected with the construct pGI-Luc200 that contained the -60 to -260 region of the human insulin gene promoter upstream of the firefly luciferase gene, or pGL-Luc, a control construct lacking the insulin promoter fragment. Transfected cells were incubated in 0.5 mmol/l glucose for Min6 cells or 3 mmol/l glucose for NES2Y or NES-PDX-1 cells for 3 h or for 3 h in 0.5 mmol/l glucose followed by 3 h in 16 mmol/l glucose, as indicated. Values are shown as relative luciferase activity standardized against protein content, and each value represents an average from six replicates; error bars represent standard deviation. Each set of values has been reproduced in three separate experiments.

Our previous studies have established that NES2Y cells have defective glucose regulation of insulin gene promoter activity as a result of the lack of PDX-1 activity (16). Thus, reporter gene analysis using a DNA construct (pGI-Luc200) that contains the -60 to -260 region of the human insulin gene promoter demonstrated that transient overexpression of PDX-1 resulted in restoration of normal glucose regulation of insulin promoter activity. In the present study, insulin gene promoter activity was analyzed in the mouse β -cell line Min6, NES2Y cells, and NES-PDX-1 cells (Fig. 2) through use of the pGL-Luc200 luciferase reporter construct and the control construct pGL-Luc, which lacks the insulin promoter fragment. In Min6 cells, high glucose concentrations (16 mmol/l) stimulated a fivefold increase in insulin promoter activity compared with that of low glucose concentrations (0.5 mmol/l) and that of the control construct (Fig. 2A). In NES2Y cells, the insulin promoter failed to respond to glucose (Fig. 2B). Overexpression of PDX-1 in the NES-PDX-1 cells restored normal insulin gene promoter activity with these cells and showed a fivefold transcriptional response to glucose (Fig. 2C). These results demonstrate that PDX-1 is required for activation of the human insulin gene promoter in response to glucose. In addition to the clonal NES-PDX-1 cell line, PDX-1-dependent activation of the human insulin gene promoter in response to glucose has also been demonstrated in multiple transient transfection studies in NES2Y cells (16,23), in additional NES2Y-derived cell lines stably overexpressing PDX-1, and in NES2Y-derived cell lines overexpressing PDX-1 in combination with SUR1 or Kir6.2, or both (24).

To investigate the effect of PDX-1 on the regulation of endogenous insulin mRNA levels, Northern blot analysis was performed on RNA extracted from NES2Y and NES-PDX-1 cells incubated in media containing low or high concentrations of glucose (Fig. 3). In NES2Y cells, there was no significant difference between the insulin mRNA levels in low and high glucose concentrations (Fig. 3A). In contrast, insulin mRNA levels in the NES-PDX-1 cells were elevated fivefold in response to 20 mmol/l glucose. To ensure equal loading, filters were additionally probed for actin with levels being unaffected by glu-

cose. Levels of insulin mRNA were quantified by phosphorimaging analysis (Fig. 3B). Over a series of four separate experiments, no significant increase in insulin mRNA levels was observed in NES2Y cells in response to glucose. In contrast, the NES-PDX-1 cells consistently showed a four- to fivefold stimulation of insulin mRNA levels in response to elevated concentrations of glucose. These results, in combination with the reporter gene analyses, suggest that transcription of the insulin gene in response to glucose is dependent on the activity of the PDX-1 transcription factor.

The role of Ca^{2+} in glucose-stimulated insulin secretion is now well understood (18). However, the role of Ca^{2+} in the regulation of insulin gene transcription remains controversial (25,26). NES2Y cells provide a unique tool for the analysis of the role of Ca^{2+} in these events. As a result of the loss of functional K_{ATP} channels, the NES2Y cells are unable to regulate the influx of Ca^{2+} (18). As a consequence, NES2Y cells constitutively release insulin, as secretion is unaffected by glucose (16). This is also the case for NES-PDX-1 cells, where insulin release is constitutively high and unaffected by glucose (data not shown). In the present study, $[Ca^{2+}]_i$ were monitored by Fura-2 microfluorometry. Basal values for the resting levels of cytosolic Ca^{2+} were 109 ± 14 nmol/l ($n = 19$) in NES2Y β -cells and 74 ± 8 nmol/l ($n = 25$, $P < 0.02$) in NES-PDX-1 cells. In both NES2Y and NES-PDX-1 cells, glucose (20 mmol/l) consistently failed to raise $[Ca^{2+}]_i$. Similarly, exposure of cells to high concentrations of extracellular KCl (40 mmol/l) or caffeine (1 mmol/l) also failed to elevate $[Ca^{2+}]_i$. These data are explained by the findings that, in both NES-PDX-1 ($n = 6$) and in control NES2Y cells ($n = 10$), it was not possible to activate openings from Ca^{2+} channels under voltage clamp conditions (Fig. 4). In contrast to the lack of effects of glucose, KCl, and caffeine, Ca^{2+} mobilization from intracellular stores was initiated in both sets of cells by a combination of the agonists acetylcholine (100 μ mol/l) and ATP (100 μ mol/l). Consistent with the current findings, acetylcholine and ATP had no effect on PDX-1-binding activity, insulin gene promoter activity, or endogenous insulin mRNA levels, either in low or high glucose concentrations (data not shown).

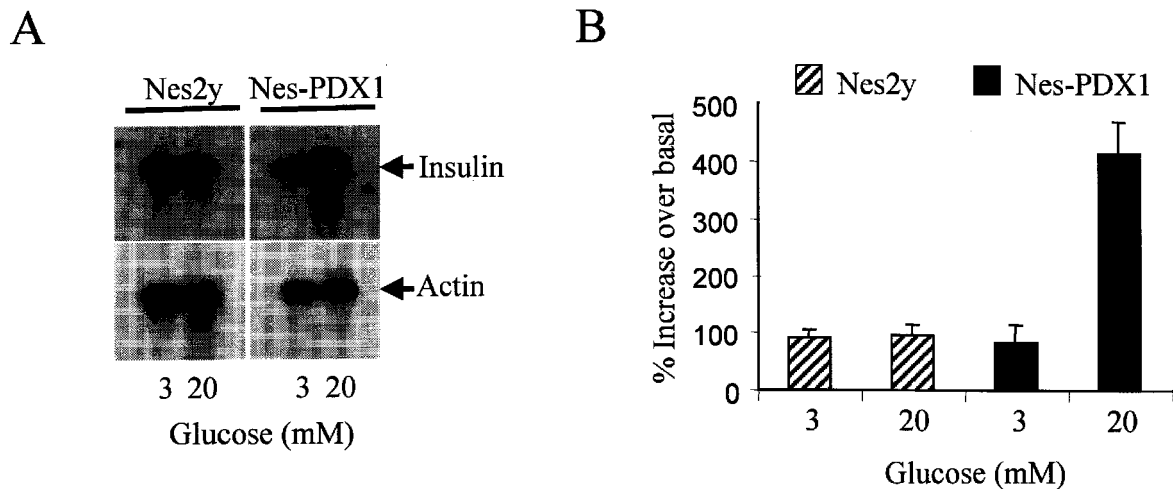


FIG. 3. Effect of glucose on endogenous insulin mRNA levels in NES2Y and NES-PDX-1 cells. A: Northern blot analysis of 3 μ g of total RNA prepared from NES2Y cells or NES-PDX-1 cells as indicated. Cells were incubated for 3 h in 3 mmol/l glucose followed by incubation for a further 5 h in 3 mmol/l glucose or 20 mmol/l glucose as indicated. Filters were probed for insulin (upper panels) and actin (lower panels). B: Phosphorimaging analysis of insulin mRNA levels. Analysis of phosphorimaged signals for insulin in NES2Y cells and NES-PDX-1 cells was treated as above. Values represent the average of four separate experiments; error bars represent standard deviation.

Whereas high concentrations of glucose stimulated PDX-1 DNA-binding activity, neither KCl nor caffeine, each of which is known to stimulate an increase in $[Ca^{2+}]_i$ in normal β -cells, was able to raise PDX-1 binding activity above that seen in low glucose conditions (Fig. 5A). Moreover, neither KCl nor caffeine was able to mimic the effects of high glucose on insulin gene promoter activity (Fig. 5B). KCl and caffeine had no effect on PDX-1 DNA-binding activity or insulin gene promoter

activity in high glucose concentrations (data not shown). In addition, glucose stimulation of PDX-1 DNA-binding activity and insulin gene promoter activity were unaffected by the presence of EGTA or the Ca^{2+} channel blocker verapamil (50 μ mol/l) (data not shown). Collectively, these data support the conclusion that glucose regulation of the insulin gene, although dependent on the activity of PDX-1, occurs independently of changes in $[Ca^{2+}]_i$.

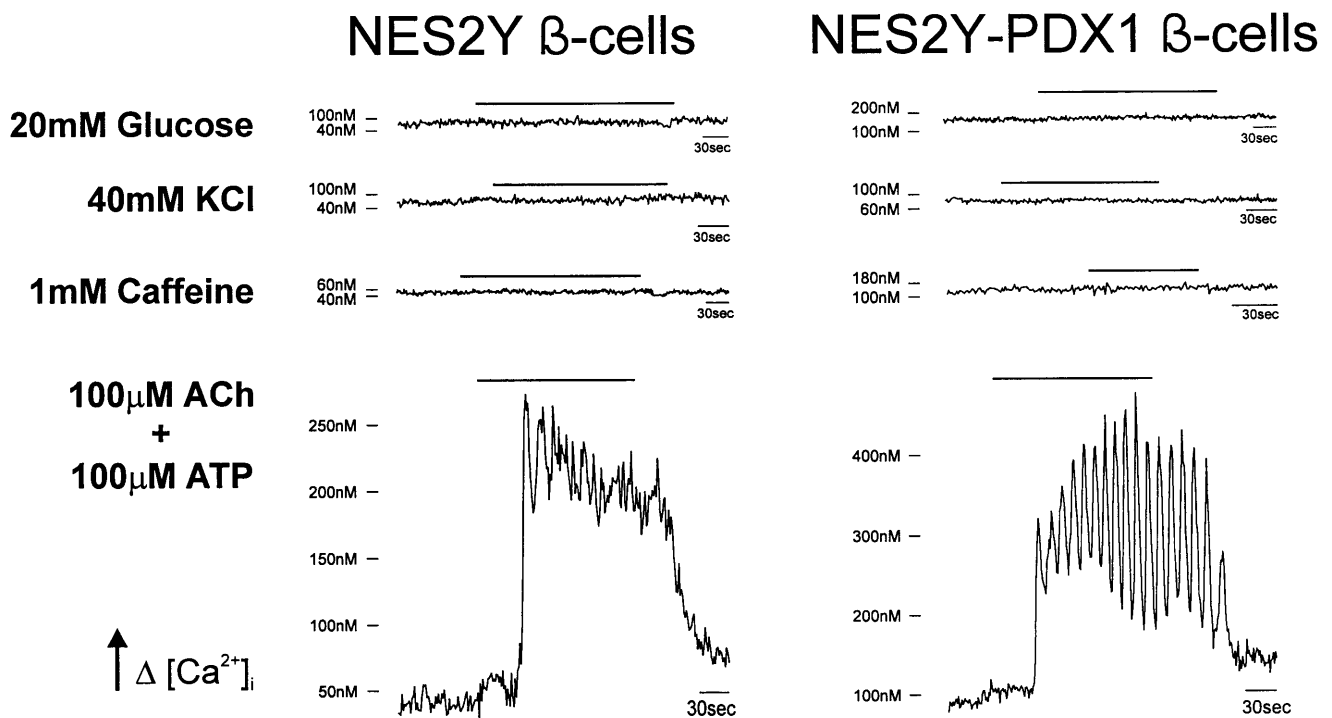


FIG. 4. Changes in $[Ca^{2+}]_i$ in NES2Y and NES-PDX-1 cells. Each panel shows a representative trace to illustrate the actions of glucose, KCl, caffeine, and a combination of 100 μ mol/l acetylcholine with 100 μ mol/l ATP on $[Ca^{2+}]_i$. In NES2Y and NES-PDX-1 cells, there were typically no observable effects of glucose ($n = 20/20$, $n = 8/8$), KCl ($n = 28/28$, $n = 4/5$), and caffeine ($n = 5/6$, $n = 4/4$), whereas a combination of acetylcholine and ATP evoked a sustained rise in $[Ca^{2+}]_i$ of 210 ± 38 nmol/l ($n = 11$) in NES2Y cells and 156 ± 65 nmol/l in NES-PDX-1 cells ($n = 9$).

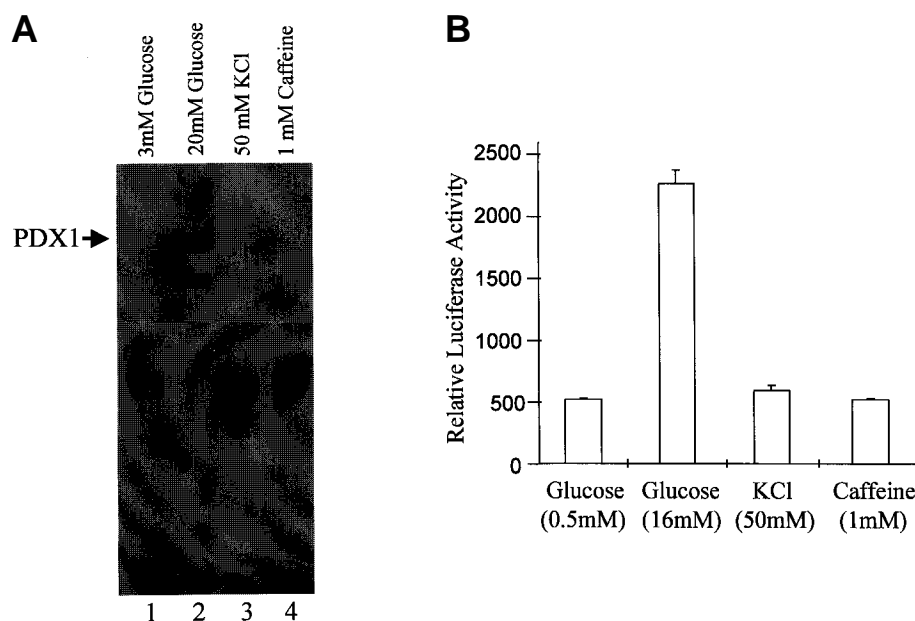


FIG. 5. Effect of Ca^{2+} -modulating agents on PDX-1 DNA-binding and insulin promoter activity. A: EMSA analysis of PDX-1-binding activity in nuclear extracts prepared from human islets of Langerhans maintained in 3 mmol/l glucose for 3 h (lane 1) or in 3 mmol/l glucose for 3 h followed by stimulation for 30 min in 20 mmol/l glucose (lane 2), 50 mmol/l KCl (lane 3), or 1 mmol/l caffeine (lane 4) by use of the A3 site of the human insulin gene promoter as probe. The arrow indicates PDX-1-binding activity. Samples were equalized for protein concentration. Comparable results were also observed in the NES-PDX-1 cells. B: Analysis of pGL-Luc200 reporter gene activity in Min6 cells. Min6 cells transfected with pGL-Luc200 were incubated in 0.5 mmol/l glucose for 3 h (lane 1), in 0.5 mmol/l glucose for 3 h followed by stimulation with 16 mmol/l glucose for 3 h (lane 2), in 0.5 mmol/l glucose for 3 h followed by 50 mmol/l KCl for 3 h (lane 3), or in 0.5 mmol/l glucose for 3 h followed by 1 mmol/l caffeine for 3 h. Values are shown as relative luciferase activity standardized against protein content. Values represent an average from six replicates; error bars represent standard deviation. Each set of values has been reproduced in three separate experiments. Comparable results were also observed in NES-PDX-1 cells.

DISCUSSION

It has previously been well established that the DNA-binding activity of the homeodomain transcription factor PDX-1 is stimulated by glucose in isolated islets of Langerhans and in β -cell lines (5–7). In addition, the activity of promoter constructs containing multiple PDX-1 binding sites was also stimulated by glucose in transfected β -cell lines (7,10,27). However, other transcription factors (e.g., RIPE3b1 [8], or one or more factors that bind to the negative regulatory element located between –258 and –270 of the human insulin promoter [9]) are also stimulated by glucose, and multiple cis-acting elements contribute to the response of the promoter to glucose (3). For these reasons, it was unclear to what extent PDX-1 contributed to the overall regulation of insulin gene expression in response to elevated concentrations of glucose. The availability of the NES2Y cell line has allowed us to address this question. The present study indicates that PDX-1 is essential for glucose regulation of the insulin promoter (Fig. 2) and insulin mRNA levels (Fig. 3).

Also, as a consequence of a defect in K_{ATP} channel activity, NES2Y and NES-PDX-1 cells are unable to modulate $[\text{Ca}^{2+}]_i$ in response to glucose (Fig. 4). This observation has allowed us to address a question concerning the role of Ca^{2+} in regulating insulin promoter activity. German et al. (25) have shown that the Ca^{2+} channel blocker verapamil (100 $\mu\text{mol/l}$) inhibited glucose effects on rat insulin promoter constructs in transfected neonatal rat islets of Langerhans. However, Goodison et al. (26) subsequently found that verapamil (100 $\mu\text{mol/l}$) had no effect on glucose-stimulated insulin mRNA production in the β -cell line HIT T15 or on rat I insulin promoter

constructs in transfected HIT T15 cells. In addition, the same authors reported that increasing extracellular Ca^{2+} concentrations from 0.4 to 5 mmol/l resulted in a marked inhibition of glucose-stimulated insulin gene expression. The present study clearly demonstrates that glucose can efficiently modulate both insulin promoter activity and endogenous insulin mRNA levels in NES-PDX-1 cells that show no change in $[\text{Ca}^{2+}]_i$ in response to glucose. These results support the conclusion that Ca^{2+} is not involved in the signaling pathway whereby glucose regulates insulin gene expression.

It is unclear why PDX-1 expression is impaired in NES2Y cells. There may be a mutation in the PDX-1 gene regulatory sequences in addition to defects associated with the K_{ATP} channel. Alternatively, the defective expression of PDX-1 may be a consequence of the inability of these cells to adequately govern $[\text{Ca}^{2+}]_i$. Although Ca^{2+} does not play a role in regulating PDX-1 activity, it may exert an influence at an early stage in the development of the endocrine pancreas (28) in controlling expression of PDX-1 or upstream regulatory genes. Based on the findings of the present study, it is clear that glucose regulation of insulin mRNA levels requires PDX-1 binding to the human insulin gene promoter. However, in the β -cell, PDX-1 also plays a role in the transcriptional regulation of other genes critical for “glucose sensing,” such as GLUT2 (12) and glucokinase (13), and it is possible that the restoration of glucose-regulated insulin mRNA levels occurs, in part, through PDX-1 effects on these other β -cell genes. The exact role of PDX-1 (and $[\text{Ca}^{2+}]_i$) in the glucose regulation of these additional genes remains to be clarified. PDX-1 binding to the insulin promoter is clearly the dominant

mechanism involved in insulin mRNA production in response to glucose. Secondary effects of PDX-1 expression that occur through the restoration of "normal glucose sensing" (i.e., normal glucose regulation of GLUT2-glucokinase expression) may also play a role in these events. Future experiments measuring glucose uptake, utilization, and oxidation in the NES2Y and NES-PDX-1 cells may well provide interesting insights into these events.

As a continuously replicating human β -cell line, NES2Y represents a valuable resource for studies on human islet cell physiology. The defect in intracellular Ca^{2+} signaling and insulin secretion can be repaired by using cell engineering approaches (24). We describe here a new cell line, NES-PDX-1, which was obtained by stably transfecting NES2Y with the homeodomain transcription factor PDX-1. This line could prove valuable in screens designed to discover drugs that could target insulin gene expression as a potential treatment for type 2 diabetes.

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