Glucose enhances insulin promoter activity in MIN6 β-cells independently of changes in intracellular Ca\(^{2+}\) concentration and insulin secretion

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Recent studies have suggested that glucose may activate insulin gene transcription through increases in intracellular Ca\(^{2+}\) concentration, possibly acting via the release of stored insulin. We have investigated this question by dynamic photon-counting imaging of insulin- and c-fos-promoter–firefly luciferase reporter construct activity. Normalized to constitutive viral promoter activity, insulin promoter activity in MIN6 construct activity. Normalized to constitutive viral promoter imaging of insulin- and c-fos promoter activity in MIN6 cells, but increased c-fos promoter activity 5-fold in AtT20 cells. Together, these results demonstrate that glucose can exert a direct effect on insulin promoter activity in islet \(\beta\)-cells, via a signalling pathway which does not require increases in intracellular Ca\(^{2+}\) nor insulin release and insulin receptor activation.

Key words: calcium, c-fos, luciferase, transcription

INTRODUCTION

Elevations in extracellular glucose concentration activate insulin gene transcription [1], though the mechanisms involved are unresolved. Studies in a murine insulinoma cell line \(\beta\)-TC3 cells [2] and foetal islet \(\beta\)-cells [3] have suggested a role for intracellular Ca\(^{2+}\) (Ca\(^{2+}\)) increases, whereas no such requirement for [Ca\(^{2+}\)] changes was evident in HIT.T15 \(\beta\)-cells [4] nor in primary islets [5]. However, recently, Leibiger et al. [6] found that, when examined at very early time points (90 min), insulin promoter activity in HIT.T15 and islet cells can be stimulated by cell depolarization with K\(^{+}\), and that activation by glucose is inhibitable by the Ca\(^{2+}\)-channel blocker nifedipine. These effects of glucose were therefore attributed to [Ca\(^{2+}\)] increases [7], resulting in the release of insulin and the activation of an insulin receptor-dependent signalling pathway. Supporting this model, \(\beta\)-cell-specific ablation of insulin receptors in transgenic mice was found to inhibit ‘first phase’ insulin secretion [8] (and see [9]), while exogenous insulin has recently been suggested as an activator of insulin secretion [10].

In addition to prompting exocytotic release of insulin, glucose-induced Ca\(^{2+}\) increases could, in principle, act on the (prepro-) insulin gene more directly. Thus, in neuronal cells, [Ca\(^{2+}\)] increases appear to be responsible for the induction of immediate early genes, including c-fos [11]. Transcriptional activation of c-fos involves multiple transcription factors acting at distinct cis-acting elements, including a serum response element (SRE) and a Ca\(^{2+}\)/cAMP response element (CRE). In addition, intronic elements have been implicated in a Ca\(^{2+}\)-dependent release from a transcriptional block [12].

To determine whether changes in [Ca\(^{2+}\)], may exert either direct or indirect (via secreted insulin) effects on insulin promoter activity, we have examined, firstly, insulin promoter regulation by glucose and [Ca\(^{2+}\)], increases in MIN6 \(\beta\)-cells, and compared this with the regulation of the c-fos promoter. We have next examined the response of the insulin promoter to exogenously added insulin, both in MIN6 \(\beta\)-cells and in fibroblasts over-expressing human insulin receptors [13]. We have also examined the regulation of the insulin promoter in heterologous AtT20 cells, in which immediate early genes, including c-fos, respond robustly to changes in [Ca\(^{2+}\)]. The results suggest that changes in extracellular glucose concentration can regulate insulin promoter activity in \(\beta\)-cells by a direct intracellular signalling pathway, which does not involve increases in [Ca\(^{2+}\)], nor the release of endogenous insulin.

EXPERIMENTAL

Materials
Sources of reagents were as previously published [14,15].

Methods
Plasmid constructs
p260Ins.Luc\(_{pp}\) was generated by subcloning the 200 nucleotide PvuII–HincII fragment (corresponding to nt −260 to −60 flanking the human insulin gene) from the insulin promoter–chloramphenicol acetyl transferase expression construct, plasmid PBC [16], into the Smal site of plasmid pGL3.Tk [14]. The latter contained cDNA corresponding to the herpes simplex virus minimal thymidine kinase (Tk) promoter fused upstream of modified firefly luciferase (pGL3 basic, Promega). Correct orientation of the insulin promoter fragment was verified by restriction with NheI (position 21 within pGL3) and BglII (position −170 of the insulin 5’-flanking region) and verified by automated DNA sequencing.

pF711fo.Luc\(_{pp}\) was generated by PCR amplification using Expand/pfu Taq\(^{TM}\) (Boehringer) of the 1656 bp cDNA fragment encoding humanized, cytosolic luciferase (Luc +, Promega) [15] with oligonucleotide primers as follows: forward, T TTT CCATGG AAG ACG CCA AAA; reverse, T TTTCCATGG

Abbreviations used: CREB, Ca\(^{2+}\)/cAMP response element binding protein; [Ca\(^{2+}\)], free intracellular Ca\(^{2+}\) concentration; CHO, Chinese hamster ovary; SRE, serum response element.

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TCT AGA ATT ACA CGG CGA (NcoI sites underlined). The amplified fragment was ligated into NcoI-restricted plasmid pF711[17]. Correct orientation was verified by restriction analysis with HindIII (+1936 with respect to the initiating codon of modified firefly luciferase) and BalI (+3 nts with respect to the c-fos NcoI site). Plasmid pCMV.Ren was obtained from Promega (termed pRL.CMV). All plasmids were purified on a CsCl gradient [18] before microinjection.

Cell isolation and culture

For experiments on the insulin promoter, MIN6 β-cells were maintained at 30 mM glucose in Dulbecco’s modified Eagle’s medium supplemented with 15% foetal bovine serum and 50 μM mercaptoethanol. Cells were transferred into Dulbecco’s modified Eagle’s medium containing 3 mM glucose (as above) 24 h before microinjection. For experiments on the c-fos promoter, MIN6 cells were cultured and maintained at 11 mM glucose, a concentration which gives approx. half-maximal stimulation of insulin secretion in this cell type [19]. Chinese hamster ovary (CHO) cells (stably over-expressing human insulin receptors) were cultured in RPMI-based medium as described [13]. Anterior pituitary-derived AtT20 cells were cultured in RPMI 1640 supplemented with 10% (v/v) foetal bovine serum.

Microinjection and photon-counting imaging

Intracellular pressure microinjection was performed using an Eppendorf 5172 transjector and a Zeiss Axiovert 25 inverted optics microscope as described previously [20]. Cell imaging was performed at 37 °C using an intensified charge-coupled device camera (Photek ICCD216; Photek Ltd., Leonards-on-Sea, Sussex, U.K.) and an Olympus IX-70 microscope with a 10 x 0.4 NA air objective [14,15]. Other details are given in the Figure legends.

Phosphorylation of CRE binding protein (CREB)

Changes in the phosphorylation state of Ser\(^{133}\) of CREB were monitored by Western analysis using a rabbit polyclonal anti-phosphoCREB antibody (Upstate Biotechnology). MIN6 and AtT20 cells were cultured for 24 h as above on 100 mm Petri dishes, and washed twice with PBS before incubation for 15 min in modified Krebs-Ringer bicarbonate medium, comprising (mM) 125 NaCl, 3.5 KCl, 1.5 CaCl\(_2\), 0.5 MgSO\(_4\), 0.5 KH\(_2\)PO\(_4\), 2.5 mM NaHCO\(_3\), 10 Heps-Na\(^+\), pH 7.4, containing 11 mM glucose, plus additions as indicated, and equilibrated with O\(_2\)/CO\(_2\) (95:5) at 37 °C. Incubation was terminated by extraction of cells in freshly prepared ice-cold buffer, comprising 50 mM Tris/ HCl, pH 7.4, 1% (v/v) Nonidet P40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 μg·ml\(^{-1}\) aprotinin, pepstatin and leupeptin, 1 mM Na\(^{+}\)VO\(_4\), 1 mM NaF and 1 μM microstatin. Extracted proteins were separated by SDS/PAGE (12% polyacrylamide) and transferred to Immobilon-\(\text{P}\)\(^{\text{TM}}\) PVDF (Sigma) membranes. Membranes were probed with anti-phosphoCREB antibody (1:1000) in PBS (20 ml) supplemented with 3% (w/v) non-fat dry milk, with constant agitation at 22 °C. Bound antibody was revealed using the ECL\(^{\text{TM}}\) (Amersham Life Sciences) Western blotting detection reagent and exposure of X-ray film. Intensity of staining was quantified with ImageQuant\(^{\text{TM}}\) image analysis software.

Ca\(^{2+}\) detection with expressed aequorin

[Ca\(^{2+}\)] was measured in MIN6 cell populations transfected using TfX-50\(^{\text{TM}}\) (Promega), as described [7,21], in Krebs-Ringer bicarbonate medium containing 3 mM glucose.

Statistical analysis

Statistical significance was calculated by Student’s \(t\)-test assuming equal variance. * \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.001\). Results are given as the means ± S.E.M. for the number of individual cells; separate preparations are given in parentheses.

RESULTS

Generation and characterization of firefly luciferase reporter constructs

In order to monitor promoter activity quantitatively and in real time in single living cells [15,22] we generated in-frame fusion constructs between the promoter regions of the insulin and c-fos genes and humanized firefly luciferase. The insulin promoter construct (plasmid p260Ins.luc\(_{\text{FF}}\)) included the region −260 to −60 nt upstream of the transcriptional start [14]. This region contains the E1, E2 and E3, and A1, A2, A3, as well as the CRE of the human insulin promoter [23]. The c-fos construct (plasmid pF711fos.Luc\(_{\text{FF}}\)) was generated by fusion of the 5’-flanking and structural elements of the human c-fos gene in-frame with luciferase cDNA. The latter was inserted at the NcoI site of c-fos exon IV, generating a construct analogous to the c-myc fusion used in the studies of Hardingham et al. [17] (Figure 1). The construct incorporated the c-fos CRE and CRE elements, as well as a potential Ca\(^{2+}\)-dependent transcriptional read-through element in intron I [12]. In each experiment, the activity of a further, independent reporter construct expressing Renilla reniformis luciferase under cytomegalovirus reporter control (pRL.CMV) was assessed to normalize for non-specific differences in basal transcriptional and translational activity [13–15,22].

After microinjection with p260InsLuc\(_{\text{FF}}\), insulin-promoter-dependent luciferase expression increased linearly in MIN6 cells maintained at 30 mM glucose, after a lag of approx. 1.5 h, for up to 8 h after microinjection (Figure 2a). In contrast, c-fos promoter-dependent luciferase expression could be detected earlier (\(t = 45\) min) and more quickly reached a stable level of luminescence (achieved between 60 min and 4 h after injection), as expected for the unstable c-fos mRNA [24] (Figure 2a). Finally, c-fos-dependent luciferase expression was strongly inducible by serum in CHO cells (firefly: \(\text{Renilla} \) luciferase ratio 5 h

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure1.png}
\caption{Construction of pF711cfos.luc\(_{\text{FF}}\)}
\end{figure}

The four exons of the c-fos gene are shown. Other details are given in the Methods section.
Role of Ca\textsuperscript{2+} and exogenous insulin in islet β-cell gene expression

Figure 2  Kinetics of firefly luciferase luminescence changes in p260Ins.lucFF and pF711cfos.lucFF microinjected into MIN6 cells

Cells were co-microinjected with (a) either p260Ins.lucFF (ins.luc 0.35 mg \(\text{ml}^{-1}\)) and pRL.CMV (0.15 mg \(\text{ml}^{-1}\)), or pF711cfos.lucFF (c-fos.luc, 0.35 mg \(\text{ml}^{-1}\)) and pRL.CMV (0.15 mg \(\text{ml}^{-1}\)), and (b) p260Ins.lucFF and pRL.CMV plus pCMV.PDX-1 (0.08 mg \(\text{ml}^{-1}\)) [14] in 2 mM Tris/HCl, pH 8.0/0.2 mM EDTA, as given in the Methods section. (a) After the addition of 1 mM luciferin, cells were imaged in complete MIN6 medium (see Methods section) supplemented with 30 mM glucose, at the times indicated (5 min integration). Cells were maintained between the acquisition of images at 37°C and in a humidified environment containing 5% CO\textsubscript{2}. Colours represent photon \(\cdot\) 5 min\textsuperscript{-1} \(\cdot\) pixel\textsuperscript{-1} as follows: blues 1–4, greens 5–7, yellow/orange 8–10, reds 11–12, white 13. (b) After microinjection, cells were incubated in the absence of luciferase cofactors at the glucose concentrations indicated (3 or 30 mM) for 6 h before imaging in the presence of 1 mM luciferin (‘luciferase’) or 1 mM luciferin plus 5 \(\mu\)M coelenterazine (‘Renilla’). The ratio of firefly:Renilla luciferase luminescence, which represents the normalized activity of the insulin promoter, was calculated off-line (blue, 0.09, red, 0.12, white 0.14). Scale bar, 30 \(\mu\)m.

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Regulation of insulin promoter activity in MIN6 β-cells

In order to observe robust effects of glucose on insulin promoter activity at early time points (6 h) after microinjection, MIN6 β-cells were co-microinjected with cDNA encoding the homeodomain β-cell transcription factor PDX-1/IPF-1 [14]. MIN6 cells were microinjected with this and reporter plasmids and immediately transferred into the experimental medium. Images were acquired 6 h after microinjection (thus providing a stimulation period of 6 h). Under these conditions, incubation in the presence of 30 mM glucose augmented insulin promoter activity (monitored as firefly:Renilla luciferase activity) by 1.6-fold (Figures 2b and 3a) compared with incubation at 3 mM glucose. These changes in glucose concentration were associated with oscillatory changes in intracellular Ca$^{2+}$ concentration (G. A. Rutter and E. Ainscow, unpublished work), which could be entirely blocked by the addition of the Ca$^{2+}$ channel blocker verapamil. In contrast, addition of verapamil (100 μM) had no significant effect on insulin promoter activity at either 3 or 30 mM glucose (Figure 3a).

The effects of increases in [Ca$^{2+}$]i up to the low micromolar range (Figure 4a), elicited with KCl or with KCl plus the L-Ca$^{2+}$-channel agonist FPL 64176 [25], were tested next. In these experiments, cells were allowed to recover for 2 h after microinjection before stimulation for 4 h with KCl or KCl plus FPL. Stimulation with KCl caused a small but significant decrease in insulin promoter activity at 3 mM glucose (Figure 3a). No effect was observed after shorter periods of stimulation with 70 mM KCl (15, 30 or 60 min stimulation, and imaging 3 h after injection; results not shown). In common with the insulin promoter, stimulation of cells with KCl or KCl plus FPL caused a decrease in c-fos promoter activity, though the activity of this promoter was also inhibited by addition of verapamil at the weakly stimulatory glucose concentration (11 mM) used.

Addition of exogenous insulin (100 nM) had no significant effect on insulin promoter activity at either of the glucose concentrations investigated (Figure 3a).

Regulation of insulin and c-fos promoter activities in AtT20 cells

We next examined whether insulin promoter activity was regulated by intracellular [Ca$^{2+}$]i manipulation in a cell type, pituitary AtT20 cells, where the c-fos promoter responds robustly to [Ca$^{2+}$]i changes [17]. AtT20 cells were stimulated with KCl alone or KCl plus FPL, as for MIN6 cells. These manoeuvres prompt increases in cytosolic (and nuclear) [Ca$^{2+}$]i to 1–2 μM [17], which are comparable with the increases provoked in MIN6 cells (Figure 4a) when account is taken of the use of fluo-3 (a calcium-sensitive fluorescent dye) rather than aequorin for these measurements.

The response of the insulin and c-fos promoters to [Ca$^{2+}$]i changes was examined in AtT20 cells using the same experimental protocols employed with MIN6 cells (see above). In AtT20 cells, insulin promoter activity was unaffected by stimulation with K$^+$ in the presence or absence of FPL. In contrast, c-fos promoter activity was enhanced following stimulation with KCl in the presence or absence of FPL (Figure 3b). These results with the c-fos promoter are congruent with those reported, but not quantified, in earlier studies [17].

Changes in CREB Ser$^{133}$ phosphorylation

Increases in the phosphorylation state of CREB at Ser$^{133}$ occur in response to elevations of [Ca$^{2+}$], in AtT20 cells [17] and in neurons [26]. This modification is necessary, but not sufficient, to stimulate the transactivational capacity of CREB and CRE-dependent transcription [27]. Stimulation of MIN6 cells with K$^+$ prompted an increase in the phosphorylation of CREB on Ser$^{133}$ (immunoactive band of relative molecular mass close to 44 K, Figure 4) which was considerably (> 20-fold) less in extent than that achieved in AtT20 cells. Thus, Ser$^{133}$-phosphorylated CREB was almost undetectable in unstimulated MIN6 cells. However, after stimulation with the cell-permeant cAMP analogue, CPT.cAMP, levels of phosphoCREB were more similar in AtT20...
KCl (K was determined after incubation of cells for 15 min under control conditions (C), with 70 mM insulin promoter activity [3.5-fold, from 0.0035 

cubation for 5 h in the presence of 100 nM insulin increased both

inorder to monitor the effects of added insulin on insulin release with KCl, nor blockade of insulin secretion with verapamil, had any effect on insulin promoter activity, nor its response to glucose. These results demonstrate that glucose can act through a signalling pathway leading to the stimulation of the insulin promoter which does not involve an increase in intracellular [Ca^{2+}] nor the release of insulin. Indeed, it can be calculated that under the static conditions used in the present

Figure 4 Effects of increasing [Ca^{2+}], and protein kinase A activity on CREB phosphorylation in AtT20 and MIN6 cells

(a) Effect of KCl and KCl plus FPL 64176 on [Ca^{2+}] in MIN6 cells. Populations of MIN6 cells were transfected with cDNA encoding cytosolic/nuclear aequorin [38] as described (see the Methods section) before exposure to medium containing 70 mM KCl or 70 mM KCl plus 3 μM FPL 64176. [Ca^{2+}] was calculated from the observed luminescence changes as described [7]. Phosphorylation of CREB Ser^{133} in (b) AtT20 and (c) MIN6 cells. The extent of phosphorylation was determined after incubation of cells for 15 min under control conditions (C), with 70 mM KCl (K') or 0.5 mM CPT-cAMP (cAMP) as described in the Methods section. Cell extracts (15 μg of protein) were separated by SDS gel electrophoresis and probed with anti-phospho-(Ser^{133})CREB antibodies. Images were quantified to reveal intensities of CREB phosphorylation (M=44 K band; c.p.m. x 10^4): control, K' and CPT-cAMP respectively of 128, 418 and 617 for AtT20 cells, and 5.0, 18.1 and 119 for MIN6 cells. The upper (48 K) band is likely to correspond to phospho-CRE modulator [39].

and MIN6 cells, suggesting that the total amount of CREB (phosphorylated and unphosphorylated) may be within a factor of 5 in AtT20 and MIN6 cells.

DISCUSSION

We have used luminescence imaging of multiple luciferase reporters to assay insulin and c-fos promoter activity dynamically in single living cells. This method provides considerable sensitivity, dynamic range and the ability to follow both increases and decreases in promoter activity [22,28].

Regulation of c-fos promoter activity by Ca^{2+} in islet β-cells

Evidence implicating an increase in [Ca^{2+}], in the induction of c-fos gene expression in neuronal cells is considerable [29]. In contrast, the role of Ca^{2+} in the control of c-fos gene expression in β-cells is less clear. However, Susini et al. [30] have recently shown that increases in extracellular glucose have no effect on c-fos transcription in control cells, but enhance the inductive effect of CPT-cAMP on c-fos and other immediate early genes. This effect of glucose apparently involves increases in [Ca^{2+}], as it is inhibitable by the Ca^{2+} channel blocker nifedipine. One explanation is that this may be due to a synergistic effect of the two agents to enhance CREB phosphorylation. In the present studies, increases in [Ca^{2+}], capable of activating the c-fos promoter in neuronal AtT20 cells ([17], and Figure 3b), were ineffective in islet β-cells, and actually inhibited the c-fos promoter after stimulation with KCl plus FPL (Figure 3b). Nevertheless, blockade of Ca^{2+} channel activity with verapamil, which is predicted to lower [Ca^{2+}] at the weakly stimulatory glucose concentration (11 mM) at which these studies were performed, may argue for some Ca^{2+}-dependence of the c-fos promoter in the β-cell. However, a further explanation for the difference between the response of the c-fos promoter to Ca^{2+} in these two cell types may be low levels of a key transcription factor responsible for activation of this promoter. We demonstrate here that both low (total) levels of CREB and a lower extent of phosphorylation on Ser^{133} are likely to contribute to the poor responsiveness to [Ca^{2+}], increases of the c-fos gene in the β-cell type (Figures 4b and 4c). Nevertheless, the absence of other neuron-specific transcription or accessory factors, such as p62TCF/Elk-1 [31] or CREB-binding protein [27], as well as a requirement for CAMP-dependent phosphorylation [30], may also contribute to the insensitivity of the c-fos gene in the β-cell type. It follows from our observations with the c-fos promoter that the effect of glucose on insulin gene transcription is unlikely to be achieved via regulation of CREB binding, as previously surmised from promoter-deletion experiments [32].

Regulation of insulin promoter activity in islet β-cells

In these studies, we demonstrate that increases in extracellular glucose concentration in the physiological range activate transcription from the insulin promoter in MIN6 β-cells, whereas added insulin is ineffective. Furthermore, neither the activation of insulin release with KCl, nor blockade of insulin secretion with verapamil, had any effect on insulin promoter activity, nor its response to glucose. These results demonstrate that glucose can act through a signalling pathway leading to the stimulation of the insulin promoter which does not involve an increase in intracellular [Ca^{2+}] nor the release of insulin. Indeed, it can be calculated that under the static conditions used in the present
studies, a maximum rate of insulin release from MIN6 cells (~40 ng/10^6 cells per h) [33] would increase the concentration of insulin by <3 x 10^{-9} M in the culture dish, a value well below levels likely to be required for maximum effects of insulin. This result is consistent with the failure to observe effects of L-Ca^{2+} channel blockade on glucose-stimulated insulin promoter activity in whole islets [5], and with the absence of an effect of sulphonylureas [34] or external Ca^{2+} depletion [35] on insulin biosynthesis [9]. At the present time, the nature of the identified glucose-activated intracellular signalling pathway is unclear, though roles for phosphatidylinositol 3-kinases and stress-activated protein kinases ([36,37]; G. daSilva-Xavier, M. Dickens and G. A. Rutter, unpublished work) have both been proposed [9].

It should be stressed that a failure to observe an effect of exogenous insulin, and of inhibiting insulin secretion, does not definitively rule out a role for insulin in the regulation of the β-cell insulin promoter under some circumstances [6]. Thus, even after the removal of insulin from the medium and cell rinsing, it is not clear whether complete elimination of insulin from liganded insulin receptors is achieved. Should this be the case, then a failure to observe an additional effect of exogenously added insulin would be expected. Thus, it is possible that the reported effects of insulin in the studies of Leibiger et al. [6] might be attributable to differences in numbers of insulin receptors, their rate of dissociation from insulin, or internalization rate, in the different cell types examined. Future studies will be required to determine the number and behaviour of insulin receptors expressed in MIN6 cells and other β-cell lines.

Supporting the view that secreted insulin may alter its own transcription under some circumstances, in a simplified system in which exogenous insulin could be entirely eliminated (CHO cells expressing human insulin receptors) [13], added insulin strongly induced the insulin promoter. Important future goals will be to determine the intracellular signalling pathways through which both glucose and insulin may transactivate insulin gene expression, and the relative contributions of these pathways to overall regulation of insulin gene expression by glucose in vivo.

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