Activation of Serum Response Factor in the Depolarization Induction of Egr-1 Transcription in Pancreatic Islet β-Cells*

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The results of the current studies define the major elements whereby glucose metabolism in islet β-cells leads to transcriptional activation of an early response gene in insulinoma cell lines and in rat islets. Glucose stimulation (2–20 mM) resulted in a 4-fold increase in Egr-1 mRNA at 30 min, as did the depolarizing agents KCl and tolbutamide. This response was inhibited by diazoxide and EGTA, indicating that β-cell depolarization and Ca$^{2+}$ influx, respectively, are essential. Pharmacological inhibition of the Egr-1 induction by H89 (48%) and calmidazolium (35%), but not by mitogen-activated protein kinase/extracellular signal-regulated kinase 1 and 2 or phosphatidylinositol 3-kinase, implied that protein kinase A and Ca$^{2+}$/calmodulin pathways are involved. Deletion mapping of the Egr-1 promoter revealed that the proximal −198 base pairs containing two serum response elements (SREs) and one cAMP-response element retained the depolarization response. Depolarization resulted in phosphorylation of cAMP-response element-binding protein, yet partial inhibition by a dominant negative cAMP-response element-binding protein, along with a robust response of a cAMP-response element-mutated Egr-1 promoter suggested the presence of a second Ca$^{2+}$-responsive element. Depolarization activation of 5XSRE-LUC and serum response factor (SRF)-GAL4 constructs, along with activation of SRF-GAL4 by co-transfection with constitutively active calmodulin kinase IV and protein kinase A, and binding of Ser$^{103}$-phosphorylated SRF in nuclear extracts, indicated that the SRE/SRF complexes contribute to the Ca$^{2+}$-mediated transcriptional regulation of Egr-1. The results of the current experiments demonstrate for the first time SRE-dependent transcription and the role of SRF, a transcription factor known to be a major component of growth responses, in glucose-mediated transcriptional regulation in insulinoma cells.

Pancreatic islet β-cell mass can be regulated by multiple stimuli, including growth factors and nutrients. The ability to produce insulin is proportional to the islet mass that is defined by a balance between rates of proliferation and apoptosis. Several growth factors have been shown to stimulate pancreatic β-cell replication (1, 2). During pregnancy, insulin resistance results in a marked increase in β-cell mass, and growth hormone and prolactin treatment have been shown to increase β-cell proliferation (3–5). Other growth factors, including insulin-like growth factor 1, hepatocyte growth factor, and nerve growth factor, have also been implicated in β-cell mitogenesis and differentiation (1, 6–8). Nutrients including glucose and certain essential amino acids have been shown to increase β-cell proliferation and increase islet β-cell mass (1). In insulin resistance states, such as obesity for example, impaired insulin-mediated glucose disposal results in increased plasma glucose levels, and this mechanism is thought to be essential for the hyperinsulinemic response of β-cells.

Glucose induces pleiotropic effects in islet β-cells, and these effects are potentially mediated by activation of multiple intracellular signaling pathways. Glucose metabolism results in the alteration of ATP/ADP levels leading to closure of nucleotide regulated K$^{+}$ channels (KATP). KATP channel inhibition results in depolarization and activation of voltage-dependent Ca$^{2+}$ channels. Ca$^{2+}$ influx has been implicated in the activation of calmodulin (CaM)$^{1}$ kinases (CamKα) II and IV, protein kinases A (PKA) and C, extracellular signal-regulated kinase mitogen-activated protein kinase, and phosphatidylinositol 3-kinase in islet β-cells (9–12). The relationships between activation of these pathways and growth responses induced by glucose have not been defined.

Changes in gene expression profiles that result from the activation of these signaling pathways are responsible for the adaptation of β-cell mass to physiological and pathological states. The specific expression profile includes the induction of immediate early genes (IEGs). IEGs are rapidly activated by initiation of signal transduction pathways, and their activation does not require protein synthesis (13). The signaling pathways for induction of IEGs exhibit considerable stimulus and tissue specificity and in general involve activation of kinase/phosphatase cascades (13). Often, IEGs are transcription factors that in turn activate expression of downstream target genes generating distinct biological responses by inducing specific long term programs of gene expression (13). The IEG responses are not restricted to genes that control cell cycle reentry but also include genes that participate in other biological processes. IEGs are universally expressed, and the signal transduction pathways leading to activation of these genes have been extensively studied in numerous tissues.

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1 The abbreviations used are: CaM, calmodulin; CaMK, Ca$^{2+}$/calmodulin-dependent kinase; PKA, protein kinase A; IEG, immediate early gene; AP-1, activator protein-1; SRE, serum response element; CRE, cAMP-response element; SRF, serum response factor; PK, forskolin; DMEM, Dulbecco’s modified Eagle’s medium; PBS, fetal bovine serum; PCR, polymerase chain reaction; CREB, cAMP-response element-binding protein; bp, base pair(s); KCREB, killer CREB; PMA, phorbol 12-myristate 13-acetate.
Glucose and other nutrients have been shown to transcriptionally activate several IEGs, including Egr-1 in insulinoma cell lines and pancreatic islets, in a Ca\(^{2+}\)-dependent manner (14, 15). Egr-1 is a zinc-finger DNA binding transcription factor that is expressed in multiple tissues and is induced by diverse stimuli of which the best studied examples have been the treatment with peptide mitogens (16–18). Egr-1 mRNA has been shown to be increased by treatment with glucose, cAMP, and several glucocorticoids in insulinoma cells and rat islets (14, 15). The promoter elements involved in Egr-1 transcription by depolarization have not been defined. The genomic 5′-flanking sequence of the murine Egr-1 contains activator protein-1 (AP-1) sites, a cAMP-responsive element (CRE), and serum response elements (SRE). The cAMP-responsive CRE has been implicated in Ca\(^{2+}\) influx induction of the glucagon gene in pancreatic β-cells (19). CRE was also essential for the induction of c-fos by the combination of glucose and glucocorticoids in INS-1 cells (19). To our knowledge, nothing is known about SRE-dependent transcription of any gene in pancreatic islet β-cells.

In the current studies, we sought to define the mechanisms whereby glucose-induced depolarization and Ca\(^{2+}\) influx activates Egr-1 transcription in insulinoma cells. We describe the signal transduction pathways, trans-activating factors, and the cis-regulatory elements involved in this process. We demonstrate that β-cell depolarization triggers a cascade of events in which Ca\(^{2+}\) influx leads to the activation of PKA and Ca\(^{2+}/\)CaM. The initiation of these signaling pathways leads to activation of CREB and SRF, a transcriptional factor involved in growth responses. This activation results in Egr-1 induction predominantly through five SRE elements in its proximal promoter. These studies for the first time link the potential role of SRE-dependent transcription as a mechanism for glucose effect in pancreatic β-cell mass.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—Phorbol 12-myristate 13-acetate, potassium chloride, 3-O-methyl glucose, 2-deoxyglucose, mannose, forskolin (FR), diazoxide, actinomycin D, EGTA, b-mercaptoundecanol, and sucrose were obtained from Sigma. PD898059, H89, calmidazolium, staurosporin, LY294002, and wortmannin were obtained from Biomol (Plymouth Meeting, PA).

**Cell Culture Conditions**—The MIN6 insulinoma cell line was obtained from Y. Oka (20) and was maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mM glucose and supplemented with 15% heat-inactivated fetal bovine serum (FBS) in humidified 5% CO\(_2\), 95% air at 37 °C. TC6-F7 and MIN6 cells were used between passages 16–42 and 26–35, respectively. For the analysis of expression, cells that were 70–80% confluent were switched to regular DMEM containing 2 mM 26–35, respectively. For the analysis of expression, cells that were treated with peptide mitogens (16–18). Egr-1 mRNA has been shown to be increased by treatment with glucose, cAMP, and several glucocorticoids in insulinoma cells and rat islets (14, 15). The promoter elements involved in Egr-1 transcription by depolarization have not been defined. The genomic 5′-flanking sequence of the murine Egr-1 contains activator protein-1 (AP-1) sites, a cAMP-responsive element (CRE), and serum response elements (SRE). Of these elements only the CRE has been implicated in Ca\(^{2+}\) induction of the glucagon gene in pancreatic β-cells (19). CRE was also essential for the induction of c-fos by the combination of glucose and glucocorticoids in INS-1 cells (19). To our knowledge, nothing is known about SRE-dependent transcription of any gene in pancreatic islet β-cells.

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**Northern Blot Analysis and Reverse Transcriptase-PCR**—Total cellular RNA was isolated by the acid guanidium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (21). Twenty µg of total RNA were fractionated by electrophoresis through a 1.1% agarose gel and transferred to a nylon membrane by the capillary blotting technique. RNA was cross-linked by UV irradiation. The cDNA probes were labeled with \[^{32}P\]dCTP using the High-Prime Labeling System (Roche Molecular Biochemicals). Prehybridization (2–4 h) and hybridization were carried out at 56 °C in Church solution (22). Hybridized blots were washed once with 0.1× SSC and 1% SDS at room temperature for 15 min and twice at 54 °C for 15 min. The membranes were exposed overnight at 80 °C using intensifying screens. Messenger RNA levels were quantitated by PhosphorImager scanning (Molecular Dynamics) and were normalized to those of β-actin. For reverse transcriptase-PCR, total RNA was reverse transcribed using the Superscript Premultiplication System and random primers (Life Technologies, Inc.). PCR was done according to the manufacturer’s protocol with addition of [\(^{32}P\)]dCTP to the PCR mixture. PCR products were separated on 5% polyacrylamide gels and exposed to X-ray film and normalized to those of β-actin. The primers for Egr-1, Egr-3, and NGFR-C have been described previously (23, 24).

**Islets of Langerhans mRNA Analysis**—Primary islets of Langerhans were isolated from adult male Harlan Sprague-Dawley rats by standard collagenase digestion (25). Pancreatic islets were cultured in RPMI medium containing 5 mM glucose for 48 h and stimulated by adding glucose to a final concentration of 25 mM for 45 min.

**Plasmids**—Plasmids containing the rat Egr-1, Egr-2, Egr-3, and NGFR-C cDNAs were kindly provided by Dr. J. D. Milbrandt (Washington University School of Medicine). The plasmids containing the murine Egr-1 promoter linked to the luciferase vector pXP2 constructs were generously provided by Dr. David Cohen (Orogen Health Sciences University) and have been described in detail previously (26). The pmEgr-1200 contains 1.2 kilobase of 5′-flanking region of the mouse Egr-1 gene (GenBankTM accession number X12617). The pmEgr-510 construct included sequences −511 to −1013 bp; the pmEgrAP1 construct contains nucleotides −1 to −386 plus −868 to −1000 (from sequence X12617). Plasmids containing nucleotides −532 to +100 (prEgr-532), −198 to +100 (prEgr-198), and −100 to +100 (prEgr-100) subcloned into the luciferase reporter plasmid pGL2 (Promega) were obtained from J. Milbrandt. The cis-reporter plasmid pSRE-luc (5XSRE-luc) contains the luciferase reporter gene driven by a basic promoter element (TATA box) joined to five tandem repeats of the c-fos SRE and CRE, respectively (Stratagene). The CREB-GAL4 system includes a trans-activator plasmid that expresses a fusion protein containing the activator domain of CREB fused to the DNA binding domain of GAL4 (residues 1–147) (CREB-GAL4) (Stratagene), cotransfected with a reporter gene containing a synthetic promoter with five tandem repeats of the yeast GAL4 binding sites that control expression of the luciferase gene pRc-CMV (Stratagene). The SRF-GAL4 fusion vector and a constitutively active form of CaMKIV (CaMKIVCT) were a gift from Dr. Michael Greenberg and have been described previously (27). The plasmid containing the catalytic unit of PKA (pFC-PKA) was purchased from Stratagene. The killer CREB (KCReB) construct was kindly provided by Dr. Richard Goodman and has been described previously (28). Briefly, KCReB contains an amino acid substitution within the leucine zipper that results in KCReB-CREB heterodimers with no DNA binding function. The pRK-TK control vector contains the herpes simplex virus thymidine kinase promoter upstream of the Renilla luciferase (Promega).

**Transient Transfections—MIN6 cells were transfected by LipofectAMINE and Plus Reagent (Life Technologies, Inc.) using the suggested amounts of DNA according to the manufacturer’s protocol. Briefly, 1 × 10\(^5\) cells were plated in 6-well plates 4 days prior to transfection. Cells at about 80% confluence were transfected by mixing the above DNA described in the figure legends, and a lipid mixture containing a 1:2 ratio of LipofectAMINE and Plus Reagent in 1 ml of OPTI-MEM medium (Life Technologies, Inc.). Following 4 h of incubation, 1 ml of DMEM containing 5 mM glucose and 2% serum was added to the cells. After 12 h, the medium-DNA complexes were replaced by preincubation medium containing DMEM with 5 mM glucose, 2% FBS, and the cells were left for 24 h. At the end of the 24 h the specific stimulating agent was added to the medium, and the cells were harvested 6 h later. For the overexpression experiments with pFC-PKA and CaMKIVCT, MIN6 cells were transfected as above, followed by incubation in DMEM containing 5 mM glucose and 2% FBS for 30 h until harvesting. Total DNA was maintained constant in all the transfection experiments by using the empty vector of the respective cDNA to be overexpressed. To correct for differences in transfection efficiencies, 1 ng of pRL-TK Renilla luciferase plasmid was simultaneously transfected. All results are normalized for transfection efficiency and expressed as the ratio of the firefly to Renilla luciferase.

**Electrophoretic Mobility Shift Assay**—The sense strand sequences of the oligonucleotides used are: c-fos SRE, GGAATTCATATAGGACGAGGAGCAGGAGGGTT; c-fos CRE, GGAATTCATATAGGACGAGGAGCAGGAGGGTT; prEgr-198, GCGCGCGCCCTTTATAGGAGTTGGCCA; Egr-1SRE#3, TGCCGATGCATATGAGGAGGAGGAC; Egr-1SRE#5 (most distal), GAAACCACATTTAGGAGGAGGACGAGGCC; Egr-1SRE#3, GGCGCGCCCTTTATAGGAGTTGGCCA; Egr-1SRE#2, CTTCCCTCCTATATAGGAGGCTTTC; Egr-1SRE#1, CTTCCCTCCTATATAGGAGGCTTTC; Egr-1SRE#1, CTTCCCTCCTATATAGGAGGCTTTC; Egr-1SRE#1, CTTCCCTCCTATATAGGAGGCTTTC; Egr-1SRE#1, CTTCCCTCCTATATAGGAGGCTTTC.
Nuclear extracts from MIN6 cells cultured under regular DMEM containing 25 mM glucose were prepared essentially as described previously (29), and protein content was determined using the Bio-Rad protein assay kit with bovine serum albumin as a standard. Lysates (5 μg of protein) were incubated in a final volume of 20 μl containing binding buffer (final concentrations: 1 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5), 200 ng of poly(dI-dC), and double-stranded γ-32P-oligonucleotide. Binding reactions proceeded at room temperature for 30 min and were analyzed by nondeaturing polyacrylamide electrophoresis followed by autoradiography.

In assays where antibodies directed against SRF (1:10) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and SRF phosphorylated at Ser103 (1:10) were tested, these were preincubated with the nuclear extracts for 20 min at room temperature (30).

Luciferase Assay—Cell lysis was performed using 400 μl of passive lysis buffer (Promega). Firefly and Renilla luciferase were measured by the dual luciferase reporter assay system (Promega) using 20 μl of cell lysate. Luciferase activity was measured in a Monolight 2010 luminometer.

In Vitro Mutagenesis—Because of the absence of an A in pmEgr-1200, the pmEgr-1.2 64 CRE construct was made by subcloning the 1.2-kilobase insert from pmEgr-1.2 into pBluescript SK(+). Mutagenesis was performed in the pBluescript SK(+)-constructed vector. The CRE mutation was performed by oligonucleotide-directed mutagenesis without the mutagenic selection method (31). In brief, single-stranded uracil-containing DNA template was obtained by transforming CJ236 and growing overnight in TY medium containing 50 μg/ml of kanamycin. The resulting plasmid was transformed into competent E. coli JM109 (32). Double-stranded template was obtained by primer extension and T4 DNA ligase. Finally, JM109 competent cells were transformed with 1 μl of the primer extension reaction. The presence of the mutation was confirmed by sequencing.

Western Blot—Cells were lysed with buffer containing 1× phosphate-buffered saline, 0.1% SDS, 0.01% dithiothreitol, and half a tablet of “Complete” protease inhibitor mixture (Roche Molecular Biochemicals). After boiling, proteins were separated by electrophoresis through 10% polyacrylamide, 0.1% SDS gels and transferred to polyvinylidene difluoride membranes. Membranes were incubated overnight at room temperature in blocking buffer containing 0.2% Tween (Tropix) and 1:1000 Tween 20. Subsequently, the membranes were hybridized at 4 °C overnight in blocking buffer containing the CREB and the phospho-CREB antibody with the dilutions recommended by the manufacturer (New England Biolabs). After three washes at room temperature the membranes were incubated in secondary horseradish peroxidase antibody for 1 h. After washing for 1 h, immunodetection was performed with ECL Western blotting Detection System (Amersham Pharmacia Biotech) following the manufacturer's protocol.

RESULTS

Induction of Egr-1 mRNA by Glucose in βTC6-F7 and MIN6 Insulinoma Cell Lines and in Rat Islets—To establish a model system to study the transcriptional regulation of Egr-1 in pancreatic β-cells, insulinoma cell lines that have been shown to secrete insulin in response to glucose in the physiological range were investigated (20). Following preincubation of βTC6-F7 cells for 20 h in 2 mM glucose, the addition of glucose to a final concentration of 25 mM resulted in increased Egr-1 mRNA levels between 30 and 60 min (4-fold, p < 0.01) that returned to basal levels by 2 h (Fig. 1A). Similar responses to glucose stimulation were also noted in MIN6 insulinoma cells and in primary cultures of isolated rat pancreatic islets (data not shown), and these responses were shown to be glucose dependent (Fig. 1B). The half-maximal response of Egr-1 transcription is of the same range as that observed for insulin secretion in this cell line (20). Egr-2 (NGFI-B or Krox 20) mRNA, but not Egr-3 mRNA, was also induced by glucose under the same conditions (data not shown). Mannose, the metabolizable epimer in position 2 of glucose, increased expression of Egr-1 mRNA, whereas nonmetabolizable sugars 2-deoxyglucose or 3-O-methylglucose had no effect (data not shown). These results are similar to those previously reported and confirm that glucose metabolism is required for Egr-1 induction (15).

Glucose metabolism is known to have several effects, the major one being depolarization through inhibition of KATP channels. Egr-1 mRNA was induced in insulinoma cells by tolbutamide and KCl, agents known to depolarize islet β-cells, with the same time course as that following glucose stimulation (data not shown), and this induction was Ca2+-dependent (Fig. 1C). To determine whether glucose induction of Egr-1 resulted solely from inactivation of KATP channels or additionally through increased glycolytic intermediates insulinoma cells (βTC6-F7) were treated with glucose in the presence of diazoxide, an agent that hyperpolarizes the cell through activation of islet KATP channels. Treatment with this agent resulted in complete inhibition of Egr-1 mRNA induction (Fig. 1D), indicating that this response to glucose was solely dependent on depolarization and subsequent Ca2+ influx. Activation of PKA by cAMP has been shown to be one of the major regulators of c-fos transcription in INS-1 insulinoma cells (19).

In the current experiments, to evaluate the effect of PKA activation on Egr-1, MIN6 cells were treated with forskolin. This treatment resulted in similar induction of Egr-1 mRNA, and the response to both stimuli was greater than that to either alone (Fig. 1D).

Effects of Pharmacological Agents on Glucose Induction of Egr-1 mRNA Expression—Specific pharmacological inhibitors were utilized to begin to define the signal transduction pathways involved in glucose and depolarization induction of Egr-1. The CaM and PKA pathways were assessed by the addition of the CaM inhibitor calmidazolium (10 μM) or the PKA inhibitor H89 (20 μM) (33, 34). Calmidazolium and H89 independently reduced the glucose induction of Egr-1 mRNA to 70% (p < 0.01) and to 56% (p < 0.01), respectively (Table I). Simultaneous inhibition of the PKA and CaM pathways resulted in a combined suppression to 40% of control (p < 0.001). Glucose has been shown to activate the extracellular signal-regulated kinase 1/extracellular signal-regulated kinase 2 mitogen-activated protein kinase pathway in a Ca2+-dependent manner (9, 10). The specific mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 and 2 inhibitor PD98059 (100 μM) used at concentrations known to inhibit these enzymes in other systems had no significant effect on Egr-1 mRNA induction by glucose (34–37) (Table I). Staurosporin, a known inhibitor of protein kinase C, did not inhibit Egr-1 induction. Interestingly, both phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 resulted in small but significant (p < 0.01) enhancements of Egr-1 mRNA induction. Taken together, these results suggested that glucose induction of Egr-1 is a Ca2+-dependent process and that the Ca2+ effects are mediated at least in part by PKA and Ca2+/CaM pathways.
ment at concentrations from 1 nM to 1 μM (data not shown), suggesting that insulin signaling through the insulin receptor is not involved in depolarization activation. The plasmid pmEgr-510 had the 5' end of the promoter containing the AP-1 elements deleted but still retained five SREs and a CRE. Transfection of this plasmid resulted in diminished basal activity relative to that of pmEgr-1200, suggesting that upstream elements may also play a role in the transcriptional regulation of this gene. This plasmid pmEgr-510 did retain the same fold induction to KCl and PMA, however. Basal expression and KCl and PMA induction, were all abolished when a construct lacking the SRE and CRE elements (pmEgrAP1) was employed. The results of these promoter deletion experiments indicated that the proximal -510 bp of the Egr-1 promoter contains the elements required for depolarization induction.

To further define the promoter elements required for induction, several additional deletion constructs of the -532 bp sequence derived from the rat Egr-1 promoter were tested (the proximal -532 bp of the mouse and rat Egr-1 genes are ~93% conserved, with a 100% similarity of the CRE and SREs). As shown in Fig. 2B, prEgr-532 contains five SREs and one CRE. This plasmid was activated by KCl and by FK, and the effect was additive in the presence of both these agents (2-, 2.3-, and 3.3-fold, p < 0.0001, p < 0.02, and p < 0.005). Treatment of the cells with PMA resulted in a 3.3-fold (p < 0.005) induction. Deletion of the three distal SREs (prEgr-198) resulted in a

![Fig. 1. Effects of glucose, KCl, tolbutamide, and forskolin on Egr-1 mRNA expression in insulinoma cells. A, time course for glucose-induced expression of Egr-1 mRNA in βTC6-F7 cells. Glucose stimulation of βTC6-F7 cells was obtained by adding glucose to a final concentration of 25 mM after a 24-h preincubation (time 0) in regular DMEM containing 2 mM glucose. Messenger RNA was isolated as described under “Experimental Procedures” at the indicated time points. The upper panel shows a representative Northern blot hybridization for Egr-1 and β-actin. The lower panel shows quantitative analysis performed by PhosphorImager scanning (Molecular Dynamics) that was normalized to β-actin levels. The data are expressed as mean ± S.E. of the fold induction over the RNA levels at time 0 (end of the preincubation period). The results are expressed as mean values ± S.E. of three independent experiments done in triplicates (*, p < 0.001). B, dose dependence of glucose induction of Egr-1 mRNA in βTC6-F7 cells. Cells were preincubated as described in A and stimulated for 45 min by adding glucose to the medium to the final concentrations indicated. Total RNA, Northern hybridization, and quantitation was obtained as described above. Results are expressed as a fold induction over the levels at the end of a 20-h preincubation. Data are shown as the mean (± S.E.) of three independent experiments done in duplicate (*, p < 0.02) (**, p < 0.001). C, depolarizing agent induction of Egr-1 mRNA. βTC6-F7 cells were preincubated as described in A, followed by incubation for 45 min with KCl and tolbutamide (TOLB). EGTA was added to the medium where indicated 5 min prior to stimulation. All the panels show a Northern hybridization for Egr-1 and β-actin representative of three experiments done in duplicate. D, K-ATP inward rectifying channel activators (Diazoxide) and PKA effects on Egr-1 mRNA induction. βTC6-F7 cells were preincubated (LG) as described followed by a 45-minute stimulation with 10 μM FK, 25 mM glucose (HG), or both (HG+FK). Diazoxide (0.6 mM) (D) was added to the culture medium 30 min prior to glucose. Northern hybridization for Egr-1 and β-actin is representative of three experiments done in duplicate.]

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**EGR-1 Transcription in Pancreatic Islet β-Cells**

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βTC6-F7 cells were preincubated for 20 h in regular media containing 2.0 mM glucose, then stimulated for 45 min in the presence of regular medium containing 25 mM glucose and the indicated pharmacological inhibitors. Pharmacological inhibitors were added to the media 1 h prior to stimulation with glucose. Total RNA was isolated as described under “Experimental Procedures.” Egr-1 mRNA levels were obtained by PhosphorImager scanning (Molecular Dynamics) and were optimized to those of β-actin. The results are expressed as mean (± S.E.) of six independent experiments. *, p < 0.02; **, p < 0.01; ***, p < 0.001.

| % of the response to 25 mM Glucose ± S.D. |
|------------------------------------------|
| 25 mM Glucose                           |
| 10 μM Calmidazolium                     |
| 20 μM H89                               |
| 20 μM H89 + 10 μM Calmidazolium         |
| 100 μM PD98059                          |
| 0.3 mM Staurosporin                     |
| 0.2 μM Wortmannin                       |
| 50 μM LY294002                          |

Further Assessment of the Role of the CRE Element and CREB Phosphorylation in Depolarization Induction of Egr-1—Transcriptional activation of the c-fos gene by Cu2+ and cAMP in neuronal cells is mediated by CREB phosphorylation of Ser133 (13). To further define the process whereby depolarization activates the CRE of the Egr-1 promoter, CREB phosphorylation was assessed in MIN6 cells following depolarization. Western blotting was performed using an antibody specific for CREB phosphorylated at Ser133 as shown in Fig. 3A, both KC and FK treatment induced CREB phosphorylation on Ser133 within five minutes. High glucose resulted in an increase in CREB phosphorylation after 30 min. The PKA inhibitor H89 diminished both the glucose and the forskolin effect on CREB phosphorylation, although the magnitude of the inhibitory effect of H89 on forskolin may have been less, as there appeared to be a lower quantity of CREB protein. The total amount of CREB protein did not differ with any of the agents tested, as indicated by using an antibody to nonphosphorylated CREB. These results are consistent with glucose-induced depolarization and Ca2+ activation of CREB, in part by a PKA-dependent mechanism.

To assess the transcriptional activity of CREB following depolarization, the induction of Egr-1 was assessed in the presence of a dominant inhibitor of CREB function (KCREB) (28). Co-transfection of the pmEgr-1200 construct along with KCREB at concentrations previously shown to inhibit CREB activation of transcription resulted in significant inhibition of the responses to glucose, KCl, and forskolin (66%, 16%, 60% and p = 0.001, p = 0.03, p < 0.02, respectively) (28, 31, 38) (Fig. 4A). The inhibition of the response to a constitutively active PKA (100%, p < 0.001 data not shown) demonstrated that KCREB was an effective inhibitor of CREB-mediated transcription.

The significant inhibition by KCREB of the glucose and KCl responses further suggested that CREB-dependent transcrip-
subjected to Western blot analysis using anti-phosphoCREB (PCREB) and anti-CREB antibodies (CREB).

Treatment with KCl resulted in a 3.2-fold response of the SRE linked to a minimal promoter was evaluated.

Experiments indicated that in addition to CREB activation of the Egr-1 promoter, MIN6 cells were cotransfected with KCREB (subcloned in pRC-RSV) (0.3 μg) or equimolar vector and pmEgr-1200 (0.5 μg). DNA concentration was maintained constant by the addition of pRC-RSV DNA. After culturing in regular medium containing 5 mM glucose and 2% FBS for 24 h, the cells were continued in the same medium or stimulated for 6 h with 25 mM glucose (HG), 10 mM FK, and 45 mM KCl.

The CRE elements contribute to Egr-1 regulation. Yet the partial inhibition strongly suggested that other factors are involved in activation of Egr-1 transcription. To more carefully define the role of CRE/CREB in transcriptional induction of Egr-1 by depolarization, a mutant Egr-1 promoter was constructed (pmEgr-1200ΔCRE) by altering the CRE element without disturbing surrounding sequences and relative spacing. The glucose effect was reduced ~50% (p < 0.02) in the pmEgr-1200ΔCRE relative to the nonmutated promoter. Remarkably the KCl response of the CRE mutant was still greater than 5-fold (Fig. 4B) and inhibited only by 14% (p < 0.04), providing further evidence that other elements are involved in depolarization activation.

Co-transfection of pmEgr-1200ΔCRE with a constitutively active PKA resulted in a response that was reduced by 63% relative to co-transfection with the nonmutated plasmid, indicating that PKA activates other elements in the promoter in addition to the CRE. The pmEgr-1200ΔCRE construct was also activated by cotransfection with a constitutively active form of CaMKIV, a known activator of both SRE- and CRE-mediated transcription (27).

**Evaluation of the SRE Elements and SRF in Depolarization Induction of Egr-1 in β-Cells**—The results of the previous experiments indicated that in addition to CREB activation of the CRE, other elements of the Egr-1 promoter were involved. To assess the effect of depolarization on SRE-dependent transcription, a luciferase plasmid containing five tandem repeats of the SRE linked to a minimal promoter was evaluated (Fig. 4). Treatment with KCl resulted in a 3.2-fold response (p < 0.0001). Treatment with forskolin resulted in 1.5-fold (p = 0.02) induction, and there was no augmentation of the KCl response by forskolin.

The SRE is continuously occupied in vivo by SRF and Ets proteins of the ternary complex subfamily (39). Activated SRF induces transcription by binding to SREs and by recruiting other factors to the SRE complex. Phosphorylation of SRF at Ser133 enhances the ability of SRF to bind the SRE (30). To demonstrate the presence of a nuclear factor binding to the SRE, nuclear extracts from MIN6 cells were incubated with a c-fos consensus SRE (Fig. 6). In the absence of nuclear extracts, band D represents migration of the free SRE oligonucleotide (lane P). At least three additional bands (A-C) were observed following incubation with nuclear extracts in the absence of competitors (lanes 1 and 7). They included two slower migrating bands (A and B) and a diffuse faster migrating complex (C). Bands A and B migrate similarly to bands identified by others as the SRE-SRF complex (band B) and the ternary complex (band A) composed of the SRE, SRF, and ELK-1 or another TCF activator of both SRE- and CRE-mediated transcription (27).

**Fig. 3. Glucose- and KCl-induced Ser133 CREB phosphorylation.** Western blotting hybridization for phospho-CREB. MIN6 cells were precultured for 24 h in DMEM containing 2 mM glucose and then stimulated with 25 mM glucose (HG), 45 mM KCl (K), and 10 mM forskolin (F, 5 min; FK, 30 min) for the indicated periods of time. The protein kinase A inhibitor H89 was added to the culture medium to a final concentration of 20 μM 1 h prior to stimulation. Protein was subjected to Western blot analysis using anti-phosphoCREB (PCREB) and anti-CREB antibodies (CREB).

**Fig. 4. The CRE elements contribute to the transcriptional activation of Egr-1 by depolarization.** A. Effects of a dominant negative mutant of CREB on depolarization induction of the Egr-1 promoter. MIN6 cells were cotransfected with KCREB (subcloned in pRC-RSV) (0.3 μg) or equimolar vector and pmEgr-1200 (0.5 μg). DNA concentration was maintained constant by the addition of pRC-RSV DNA. After culturing in regular medium containing 5 mM glucose and 2% FBS for 24 h, the cells were continued in the same medium or stimulated for 6 h with 25 mM glucose (HG), 10 mM FK, and 45 mM KCl. The data are expressed as mean ± S.E. of the fold induction over the luciferase activity at 5 mM glucose. The results are representative of three independent experiments done in triplicate. *, p = 0.04; **, p = 0.02; ***, p < 0.0001.

B. Transfection with the mutated CRE Egr-1 promoter. MIN6 insulinoma cells were transfected with 0.5 μg of pmEgr-1200 and 0.5 μg of pmEgr-1200ΔCRE as described under “Experimental Procedures.” Constitutively active PKA (pFC-PKA) (50 ng) and CaMKIV (CaMKIVCT) (50 ng) were cotransfected where indicated. Preculture and stimulation were done as described in A. The data are expressed as mean ± S.E. of the fold induction over the luciferase activity at 5 mM glucose. Values are representative of three independent experiments done in triplicate. Significance was calculated by comparing the response to the wild type. *, p = 0.04; **, p < 0.02. To control for transfection efficiency 2 ng of pRL-TK luciferase construct was used in all the experiments.
We considered whether the glucose-induced rapid transcriptional activation of Egr-1 in insulinoma cells could be secondary to glucose-induced insulin secretion, i.e., an autocrine/paracrine effect. A prominent anabolic property of insulin is its effect on gene transcription. Acting through the insulin receptor, insulin has been shown to activate IEG transcription through a Ras/Raf/mitogen-activated protein kinase-dependent pathway (45–47). This appears to be an unlikely mechanism.

**DISCUSSION**

The results of the current studies have now elucidated mechanisms whereby glucose induced depolarization and Ca\(^{2+}\) influx regulates Egr-1 transcription in insulinoma cells. Failure of glucose to induce Egr-1 in the presence of diazoxide, an inhibitor of depolarization, conclusively showed that glucose metabolism in the absence of depolarization was not sufficient to activate Egr-1 transcription. Pharmacological inhibitor studies suggested that CaM and PKA pathways are involved in the glucose response. In addition, augmentation of the response by phosphatidylinositol 3-kinase inhibitors suggested that this pathway (45–47). This appears to be an unlikely mechanism.

**EGR-1 Transcription in Pancreatic Islet β-Cells**

**Fig. 5.** The SREs are major elements responsible for depolarization induction of Egr-1. Depolarization and forskolin effects on transfected SRE elements. MIN6 insulinoma cells were transfected with 0.5 μg of SRE-LUC as described under “Experimental Procedures.” To control for transfection efficiency 2 ng of pRL-TK luciferase construct was used. After culturing in regular medium containing 5 mM glucose and 2% FBS for 24 h, the cells were continued in the same medium or stimulated for 6 h with 10 μM FK or 45 mM KCl, either alone or in the combinations shown. The data are expressed as mean ± S.E. of the fold induction over the luciferase activity at 5 mM glucose. The results are representative of three independent experiments done in triplicate. *p ≤ 0.02; **p ≤ 0.002; ***p ≤ 0.0003.

**Fig. 6.** SRF and phosphorylated Ser\(^{103}\) SRF bind the Egr-1 SREs. Nuclear extracts from MIN6 cells cultured in 25 mM glucose were used in electrophoretic mobility shift assays with a probe containing the c-fos SRE sequence (lanes 1 and 7). The individual bands representing SRE binding complexes are labeled A, B, and C. D indicates migration of free probe. Unlabeled Egr-1 SRE#5 (most distal) competitor nucleotide was either not added (lane 1) or added at 10-, 25-, or 50-fold molar excess as indicated (lane 2, 3, and 4, respectively). Competition with unlabeled mutated c-fos SRE (see “Experimental Procedures”) at 25- or 50-fold molar excess is shown in lanes 5 and 6. Preincubation of nuclear extracts from MIN6 cells with antibodies against SRF (lane 6) or phosphorylated Ser\(^{103}\) specific aSRF (lane 9) were used. Lanes 10 and 11 demonstrate the addition of two non-specific antibodies. Similar results were obtained in three different experiments.
for glucose induction of Egr-1 transcription in insulinoma cells; however, as KCl activation of Egr-1 transcription was not inhibited by inhibitors of mitogen-activated protein kinase or phosphatidylinositol 3-kinase (Table I), known mediators of insulin signaling pathways for gene transcription. Further, experiments evaluating directly the effects of insulin on MIN6 cells showed no activation of Egr-1 transcription when exogenous insulin was added at concentrations as high as 1 μM. That this insulin signaling pathway is intact in these insulinoma cells has been demonstrated by activation of Akt/PKB in response to exogenous insulin in this concentration range.2

Because the depolarization-induced activation of Egr-1 transcription occurred as early as 30 min and was independent of new protein synthesis, we assessed the role of phosphorylation of transcription factors known to activate early gene transcription in other cells. For example, in neuronal cells, depolarization induction of c-fos results for the most part through Ca2+ activation of CREB (48, 49). We initially suspected that depolarization induction of Egr-1 in pancreatic islet β-cells would be because of a similar Ca2+ activation of CREB. In fact, earlier studies of depolarization-induced gene transcription in hamster insulinoma (HIT) cells emphasized the role of activated CREB interacting with the CRE of the glucagon gene promoter (50). The current observations demonstrated that, like depolarization activation of the glucagon gene, CRE/CRESRE is also involved in depolarization-mediated transcriptional induction of Egr-1. The results of the promoter deletions and mutation of the CRE element supported this idea. Depolarization resulted in phosphorylation of CREB, and transfection of insulinoma cells with a dominant negative CREB had a significant inhibitory effect. It should be noted, however, that the small inhibition of the depolarization response by the dominant negative KCREB and the robust response of the mutated CRE construct is not surprising because the Egr-1 promoter contains five SREs in addition to the CRE. These results diverge to those described for the c-fos gene in which the CRE is a potent mediator of transcriptional activation by Ca2+ signaling (48, 49). The sum of these findings, along with the demonstration of binding of CREB to the Egr-1 promoter CRE (data not shown), indicated that Ca2+-mediated CREB-dependent transcriptional activation contributes to depolarization induction of Egr-1, although other elements in the promoter can mediate this response.

The results of the current experiments demonstrate for the first time the role of the SRE/SRF in depolarization induction of transcription in insulinoma cells. Susini et al. (14) evaluated glucose and cAMP induction of the c-fos gene in INS-1 insulinoma cells. In their study, induction of the c-fos promoter was dependent on an intact CRE and not altered following mutation of the SRE. This study differed from the present one in that c-fos was not induced by glucose in the absence of cAMP nor was the response to KCl-induced depolarization assessed. In addition, the c-fos promoter has only one SRE, whereas the Egr-1 promoter has five SREs. In the present studies, the responses of the mutated CRE plasmid pmEgr-1200ACRE and 5XSRE-LUC to depolarization demonstrated that the SREs also serve as Ca2+-response elements in this model. Similar conclusions have been made for the c-fos promoter in PC12 cells, where membrane depolarization rapidly induces the phosphorylation of SRF at Ser103, and this phosphorylation enhances the ability of SRF to bind the SRE (30). The SRE consensus sequence, CC(A/T)6GG, is bound in vitro and in vivo by SRF protein. The demonstration of transcriptional activation of SRF by the SRF-GAL4 assay, as well as that of phosphorylated SRF bound to the SRE (Fig. 6), indicated that a similar mechanism exists in insulinoma cells. The present

for glucose induction of Egr-1 transcription in insulinoma cells; however, as KCl activation of Egr-1 transcription was not inhibited by inhibitors of mitogen-activated protein kinase or phosphatidylinositol 3-kinase (Table I), known mediators of
studies do not define how Ca\(^{2+}/\text{CaM}\) activates SRF. Whereas both CaMKII and CaMKIV can phosphorylate SRF in vitro, CaMKIV is the best candidate because of its nuclear localization (51). Demonstration that transfection of insulinoma cells with a constitutively active CaMKIV also induced the activation of SRF (see Fig. 7) suggested that this kinase is likely to mediate Ca\(^{2+}/\text{CaM}\) activation of transcription.

These findings demonstrate the effect of glucose on SRE/\text{SRF}-dependent transcription in pancreatic islet \textbeta-cells. The SRF is a transcription factor early defined as one of many mediating mitogenic responses and regulating fibroblast proliferation in response to growth factors (52). Activated SRF induces transcription by binding to SREs and by recruiting accessory proteins (ternary complex factors). These accessory proteins can potentiate the transcriptional response of the SRF. However, the contribution of transcription factors that interact with SRF in SRE-dependent transcription by glucose in \textbeta-cells remains undefined. Prolonged exposure to glucose is a well recognized stimulus to \textbeta-cell hypertrophy and hyperplasia (1). The current results suggest that following exposure to glucose, ensuing \textbeta-cell depolarization and Ca\(^{2+}\) induction of SRE-mediated transcription, as well as CRE-mediated transcription, could represent important mechanisms that regulate the normal morphological and physiological changes of pancreatic islets and the changes occurring in pathological conditions such as diabetes.

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REFERENCES

1. Swenne, I. (1992) Diabetologia 35, 193–201
2. Welsh, M., Mares, J., Oberg, C., and Karlsson, T. (1993) Diabetes Metab. Res. Rev. 9, 25–36
3. Nielsen, J. H., Linde, S., Welinder, B. S., Billestrup, N., and Madsen, O. D. (1989) Mol. Endocrinol. 3, 165–173
4. Billestrup, N., and Nielsen, J. H. (1991) Endocrinology 129, 883–888
5. Billestrup, N., and Nielsen, J. H. (1991) Endocrinology 129, 45–57
6. Swenne, I., Hill, D. J., Strain, A. J., and Milner, R. D. (1987) Diabetes 36, 288–294
7. Beattie, G. M., Rubin, J. S., Otonkoski, T., and Hayek, A. (1996) Diabetes 45, 1223–1228
8. Polak, M., Scharffmann, R., Seilheimer, B., Eisenbarth, G., Dressler, D., Verma, I. M., and Poter, H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5781–5785
9. Khos, S., and Cobb, M. H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5599–5604
10. Frodin, M., Sekine, N., Roche, E., Filhoux, C., Prentki, M., Wollheim, C. B., and van Obberghen, E. (1995) J. Biol. Chem. 270, 7882–7889
11. Persaud, S. J., Wheller-Jones, C. P. D., and Jones, P. M. (1996) Mol. Endocrinol. 10, 140–146
12. 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
13. Sheng, M., and Greenberg, M. E. (1990) Neuron 4, 477–485
14. Susini, S., Roche, E., Prentki, M., and Schlegel, W. (1998) FASEB J. 12, 1173–1182
15. Josefsson, K., Sorensen, L. R., Buschard, K., and Birkenbach, M. (1999) Diabetes 48, 195–203
16. Christy, B. A., Lau, L. F., and Nathans, D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7857–7861
17. Lemaire, P., Revelant, O., Bravo, R., and Charnay, P. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4691–4695
18. Milbrandt, J. (1987) Science 238, 797–799
19. Susini, S., van Haasteren, G., Li, S. L., Prentki, M., and Schlegel, W. (2000) FASEB J. 14, 124–136
20. Ishihara, H., Asano, T., Tsukuda, K., Katagiri, H., Inukai, K., Anai, M., Kikuchi, M., Yazaki, Y., Miyazaki, J. I., and Oka, Y. (1993) Diabetologia 36, 1139–1145
21. Chomezynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
22. Church, G. M., and Gilbert, W. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 1991–1995
23. Mack, K. J., Yi, S. D., Chang, S., Millan, N., and Mack, P. (1995) Mol. Brain Res. 39, 140–146
24. Shao, H., Kone, D. H., Chen, L. Y., Rubin, E. M., and Kaye, J. (1997) J. Exp. Med. 185, 711–744
25. McDaniel, M. L., Colca, J. R., Katagal, N., and Lacy, P. E. (1983) Methods Enzymol. 98, 182–200
26. Cohen, D. M., Gullans, S. R., and Chin, W. W. (1996) J. Biol. Chem. 271, 12903–12909
27. Miranti, C. K., Ginty, D. D., Haung, G., Chatila, T., and Greenberg, M. E. (1995) Mol. Cell. Biol. 15, 3672–3684
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