Activation of Elk-1, an Ets transcription factor, by glucose and EGF treatment of insulinoma cells

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Activation of Elk-1, an Ets transcription factor, by glucose and EGF treatment of insulinoma cells. Am J Physiol Endocrinol Metab 281: E1286–E1299, 2001.—Elk-1, a member of the ternary complex factor family of Ets domain proteins that bind serum response elements, is activated by phosphorylation in a cell-specific manner in response to growth factors and other agents. The purpose of the current study was to determine whether Elk-1 activation contributes to glucose-/depolarization-induced Ca2+-dependent induction of immediate early response genes in pancreatic islet β-cells. The results of experiments in insulinoma (MIN6) cells demonstrated that Elk-1-binding sites (Ets elements) in the Egr-1 gene promoter contribute to transcriptional activation of the gene. Treatment with either epidermal growth factor (EGF), a known inducer of β-cell hyperplasia, glucose, or KCl-induced depolarization resulted in Ser383 phosphorylation and transcriptional activation of Elk-1 (4 ± 0.3, P = 0.003, 2.3 ± 0.19, P = 0.002, and 2.2 ± 0.1-fold, P = 0.001 respectively). The depolarization response was inhibited by the Ca2+ channel blocker verapamil and by the MEK inhibitor PD98059 (53 ± 6 and 55 ± 0.5%, respectively). EGF-induced activation of Elk-1 was also inhibited by PD98059 (60 ± 5%). A dominant negative Ras produced partial inhibition (42%) of the depolarization-induced Elk-1 transcriptional activation. Transfection with a constitutively active Ca2+/calmodulin kinase IV plasmid also resulted in Elk-1 transcriptional activation. Experiments with p38, phosphatidylinositol 3-kinase, and protein kinase A inhibitors indicated that these pathways are involved in the regulation of Elk-1 activation. Elk-1 activation contributes to glucose- and depolarization-induced Ca2+-dependent induction of immediate early growth response genes in pancreatic islet β-cells. Furthermore, the results demonstrated a convergence of nutrient- and growth factor-mediated signaling pathways on Elk-1 activation through induction of Ras/mitogen-activated protein kinase ERK-1 and -2. The role of these pathways in the glucose-induced proliferation of islet β-cells can now be assessed.

Recent success with human islet transplantation in diabetic patients underscores the need for adequate numbers of islet β-cells for this therapy. A compensatory increase in pancreatic islet β-cell mass occurs with insulin resistance states, yet the mechanisms involved in this response are little understood (14). Plasma glucose appears to be a major component in this response, as prolonged hyperglycemia in experimental animal models leads to increase in β-cell mass (4). Glucose induces pleiotropic effects in islet β-cells that are depolarization and Ca2+-dependent, and these effects are potentially mediated by activation of multiple intracellular signaling pathways. Depolarization-induced Ca2+ influx has been implicated in the activation of Ca2+/calmodulin (CaM) kinases II (CaMKII) and IV (CaMKIV), protein kinases A (PKA) and C (PKC), extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) (MEK) (16, 32), and phosphatidylinositol 3-kinase (PI 3-kinase) in islet β-cells (16, 23, 27, 32). For glucose-induced depolarization and insulin-like growth factor (IGF) treatment of insulinoma cells, studies with pharmacological inhibitors have implicated the MAPK/ERK and PI 3-kinase pathways in proliferative responses (19). The relationships between these activated pathways, their downstream targets, and glucose-regulated growth responses have not been defined (35).

A prominent component of the cellular response to mitogenic stimuli is the rapid and transient transcriptional activation of a group of genes termed immediate early genes (IEGs) (38), most notably c-fos and Egr-1 (17). This rapid response of IEG transcription to mitogenic stimuli suggests that the products of these genes likely have a regulatory role in the cellular responses to growth factors (10). Like growth factors, glucose, peptide hormones (glucocretins), and other nutrients have been shown to activate transcription of several IEGs including Egr-1 in insulinoma cell lines and pancreatic islets (22, 39). 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 (7, 22, 25, 30). Recently, in an...
effort to define the signal transduction pathways involved in glucose regulation of Egr-1 transcription, we found that glucose-induced β-cell depolarization triggers a cascade of events in which Ca\(^{2+}\) influx leads to the activation of PKA and CaM pathways (3). The initiation of these signaling pathways leads to activation of cAMP response element (CRE)-binding protein (CREB) and serum response factor (SRF), transcription factors involved in growth responses. The Egr-1 induction after depolarization was shown to result predominantly through serum response element (SRE)-dependent transcription. These studies defined a role of SRF activation as a mechanism for the glucose effect on pancreatic β-cell gene expression.

SRF is a transcription factor early defined as being one of many that mediate mitogenic responses and regulate fibroblast proliferation in response to growth factors (42). Activated SRF by phosphorylation cascades induces transcription by binding to SREs and by recruiting ternary complex factors (TCFs). TCFs are members of the Ets family of transcription factors that are involved in the formation of the ternary complex with the SRF and SRE. Elk-1 is the most prominent member of the TCF family of transcription factors (33, 41). Whether Elk-1 activation contributes to SRE-dependent transcriptional responses appears to depend on the cell type and the stimulus (15).

In the present studies, we found that glucose-induced depolarization of pancreatic islet β-cells triggers a cascade of events in which Ca\(^{2+}\) influx leads to Elk-1 activation through the Ras/MEK/ERK pathway. In addition, activation of the CaMKIV pathway also results in Elk-1-dependent transcription by an ERK-dependent mechanism. Epidermal growth factor (EGF) treatment, like depolarization, also results in Elk-1-dependent transcriptional activation by a similar pathway. These studies demonstrate convergence of depolarization- and growth factor-activated mitogenic responses on Elk-1 transcriptional activation in islet β-cells.

**EXPERIMENTAL PROCEDURES**

**Chemicals.** Phorbol 12-myristate 13-acetate, KCl, verapamil, EGF, growth hormone, prolactin, and IGF-II were obtained from Sigma (St. Louis, MO). PD98059, H89, SB203580, and wortmannin were obtained from Biomol (Plymouth Meeting, PA).

**Cell culture conditions.** The MIN6 insulinoma cell line was obtained from Y. Oka (Yamaguchi University, Yamaguchi, Japan) (20) and was maintained in Dulbecco's modified Eagle's medium (DMEM) as previously described (3). All of the experiments were performed in cells between passages 20 and 30.

**Plasmids.** Plasmids containing the murine Egr-1 promoter linked to the luciferase vector pXP2 constructs have been described in detail previously (9). The cis-reporter plasmid 5XSRF+Ets-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 lacking the Ets-binding elements (Stratagene). The Elk-GAL4 luciferase system includes a trans-activator plasmid that expresses a fusion protein containing the activator domain of Elk-1 fused to the DNA-binding domain of GAL4 (residues 1–147; Stratagene) and was 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-Luc (Stratagene). The constitutively active forms of CaMKIV (CaMKIVCT) and CaMII (CaMIIyBC) and the dominant negative forms of CaMKIV (CaMKIVK75E) and CaMII (CaMIIyBI) were gifts from Dr. T. Chatila (Washington University, St. Louis, MO) and have been described previously (31). The plasmid containing the catalytic unit of PKA (pFC-PKA) was purchased from Stratagene. The plasmids pZIP-ras Ras wild-type (WT) and pZIP-ras 15A (Ras 15A) and the vector pZIP-NeoSV were provided by Dr. C. Der (University of North Carolina) (6, 11). The plasmids encoding the MAP kinase phosphatase 1 (MKP-1) and Elk-1 (CMV-Elk-1) were kindly provided by Dr. J. Pessin (University of Iowa) and Dr. J. Schwartz (University of Michigan) (26). The pR-LK control vector contains the herpes simplex virus thymidine kinase promoter upstream of Renilla luciferase (Promega).

**Transient transfections.** MIN6 cells were transfected by lipofectamine and Plus Reagent (GIBCO-BRL) by using the suggested amounts of DNA according to the manufacturer's protocol. Briefly, 1 × 10⁵ cells were plated in 12-well plates 3 days before transfection. Cells at ~60% confluence were transfected by mixing the indicated amount of 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 media (GIBCO-BRL). After 3 h of incubation, 0.5 ml of DMEM media containing 5 mM glucose and 2% serum was added to the cells. After 12 h, the medium-DNA complexes were replaced by preincubation media containing DMEM with 5 mM glucose and 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 media, and the cells were harvested 6 h later. For the overexpression experiments with pFC-PKA, CaMKIVCT, CaMIIyBC, MEK, and MEKK, MIN6 cells were transfected as described, followed by incubation in DMEM containing 5 mM glucose and 2% FBS for 30 h until harvesting. For the experiments that include the dominant negative forms of CaMII and IV, and pZIP-ras (WT), pZIP-ras (15A), MIN6 cells were cultured as described, but the last 6 h of the experiment were in the presence of the stimulating agent. Total DNA was maintained constant in all of the transfection experiments by using the empty vector of the respective cDNA to be overexpressed. To correct for differences in transfection efficiencies, 2 ng of pRL-TK luciferase plasmid were simultaneously transfected. All results are normalized for transfection efficiency and expressed as the ratio of firefly to Renilla luciferase.

**Electrophoretic mobility shift assay.** The sense strand sequences of the oligonucleotides used were

- c-fos SRE GGATGTCATATTAGGACATCTGC
- c-fos mutSRE ACAGGATGTCATATTAGGACATCTGC
- E74 GATCTCTAGCTGAATACCGGAGAATCATCCTTGC

Nuclear extracts from MIN6 cells cultured under regular DMEM containing 25 mM glucose were prepared essentially as described previously (12), and protein content was determined using the Bio-Rad protein assay kit with bovine serum albumin as the standard.
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MAPK (Thr202 and Tyr204) antibody. The resulting immunoprecipitated overnight at 4°C with an anti-phospho-p44/42 MAP kinase (P44/42 MAP Kinase Assay Kit (Cell Signaling Technology)) was performed using the Dual-Luciferase Reporter Assay System (Promega) with the use of 20 μl of cell lysate. Luciferase activity was measured in a Monolight 3010 luminometer.

Immunoblotting. MIN6 cells were plated in six-well plates and transfected by lipofectamine and Plus Reagent (GIBCO-BRL) with 1 μg of CMV-Elk-1 as described. Thirty-six hours later, cells were preincubated in Krebs-Ringer bicarbonate-HEPES buffer (KRBBH) and 2% albumin for 1 h followed by stimulation with the indicated agents. Cells were lysed with buffer containing 1× PBS, 0.1% SDS, 0.01 M dithiothreitol, and one-half of a tablet of “Complete” protease inhibitor cocktail (Boehringer Mannheim). 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 1% BSA and adjacent Ets motifs (GGA) that have been implicated in mediating a component of their enhancer activity in other cells (41). To define a minimal promoter suitable to investigate the contribution of the Ets elements in depolarization induction of Egr-1 in pancreatic β-cells, several deleted constructs of the Egr-1 promoter were assessed. As shown in Fig. 1A, although the 1.2-kb promoter was induced ninefold (P < 0.003), deletion of the two activating protein 1 (AP-1) elements, the two proximal SRE/Ets elements, and the CRE (construct C) still retained sixfold induction (P = 0.03) by depolarization. This suggested that the distal cluster of SRE/Ets elements in construct C could be used to refine the contribution of these binding sites in the depolarization response. Parenthetically, basal activity for construct C was increased relative to the full promoter construct A, suggesting perhaps the presence of inhibitory elements in the proximal promoter.

To further determine the role of Ets elements in depolarization-induced, SRE-dependent transcription, MIN6 cells were transfected with a plasmid containing the SREs in conjunction with both clusters of Ets motifs (plasmid A) resulted in a fivefold activation (P = 0.0004). Deletion of the 3’ Ets motif (plasmid B) resulted in a 3.3-fold induction (P = 0.0002), although this response was decreased (42%) relative to construct A (P = 0.003). The plasmids containing a deletion of the 5’ Ets cluster (plasmid C) had a minimal response to depolarization (1.6-fold, P = 0.01), and this response was similar to that of the plasmid containing no Ets elements (plasmid D, 1.6-fold, P = 0.006). The results in Fig. 1, A and B, suggested that the SRE along with the Ets elements is sufficient to mediate depolarization induction of Egr-1 transcription and that the Ets elements are important components of this response.

To further determine the role of Ets elements in depolarization-induced, SRE-dependent transcription, MIN6 cells were transfected with a plasmid that contains five tandem SRE repeats (plasmid 5XSRE+Ets-Luc). Each repeat contains both the c-fos SRF and Ets-binding sites linked to a minimal promoter (Fig. 1C, inset). This plasmid was induced by depolarization (3.6-fold, P = 0.006) and by EGF (2-fold, P = 0.04), a growth factor known to activate SRE-dependent transcription and be involved in β-cell hyperplasia (24, 29). Deletion of the Ets element (5XSRE-Luc) resulted in a lack of response to either stimulus. These results further highlight the importance of the Ets elements in

RESULTS

Ets elements are necessary for depolarization induction of SRE-dependent transcription in MIN6 insulinoma cell lines. Previous studies showed that the proximal 530 bp of the Egr-1 promoter, containing five SREs, are sufficient for depolarization activation of the promoter (3). The purpose of the present studies was to determine the role of Ets-dependent transcription in this process. SREs are comprised of SRF-binding sites and adjacent Ets motifs (GGA) that have been implicated in mediating a component of their enhancer activity in other cells (41). To define a minimal promoter suitable to investigate the contribution of the Ets elements in depolarization induction of Egr-1 in pancreatic β-cells, several deleted constructs of the Egr-1 promoter were assessed. As shown in Fig. 1A, although the 1.2-kb promoter was induced ninefold (P < 0.003), deletion of the two activating protein 1 (AP-1) elements, the two proximal SRE/Ets elements, and the CRE (construct C) still retained sixfold induction (P = 0.03) by depolarization. This suggested that the distal cluster of SRE/Ets elements in construct C could be used to refine the contribution of these binding sites in the depolarization response. Parenthetically, basal activity for construct C was increased relative to the full promoter construct A, suggesting perhaps the presence of inhibitory elements in the proximal promoter.

To further determine the role of Ets elements in depolarization-induced, SRE-dependent transcription, MIN6 cells were transfected with a plasmid that contains five tandem SRE repeats (plasmid 5XSRE+Ets-Luc). Each repeat contains both the c-fos SRF and Ets-binding sites linked to a minimal promoter (Fig. 1C, inset). This plasmid was induced by depolarization (3.6-fold, P = 0.006) and by EGF (2-fold, P = 0.04), a growth factor known to activate SRE-dependent transcription and be involved in β-cell hyperplasia (24, 29). Deletion of the Ets element (5XSRE-Luc) resulted in a lack of response to either stimulus. These results further highlight the importance of the Ets elements in
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Fig. 1. Ets elements are important for depolarization induction of serum response element (SRE)-dependent transcription in MIN6 insulinoma cells. CRE, cAMP response element; AP-1, activating protein 1. A: depolarization effects on Egr-1 promoter activity. Egr-1 luciferase promoter 5'-deletion plasmids were transiently transfected as described in EXPERIMENTAL PROCEDURES with 0.2 μg of the indicated constructs. To control for transfection efficiency, 2 ng of the pRL-TK luciferase construct were used. After 24-h preincubation in regular media containing 5 mM glucose and 2% serum, cells were continued in preincubation media containing 5 mM glucose (control) or were stimulated with 45 mM KCl for 6 h. Values are expressed as the ratio of firefly to Renilla luciferase. Basal promoter activity for constructs A, B, C, and D was 1.45, 0.07, 11.7, and 0.28 arbitrary units, respectively. B: effects of KCl-induced depolarization on Egr-1 promoter deletion constructs containing sequences in the vicinity of SRE-3 and -4 as shown schematically in the inset. MIN6 cells were transfected with 0.2 μg of the indicated deletion mutants lacking one (B and C) or both (D) of the 5'- and 3'-cluster of Ets motifs (filled ovals); all were subcloned upstream of the minimal Egr-1 promoter (plasmid D in A). Cells were cultured as described in A and stimulated with 45 mM KCl. Basal activity for constructs A, B, C, and D were 11.3, 11.4, 7.2, and 2.7 arbitrary units, respectively. C: depolarization induction of c-fos SRE deletion constructs. As shown in the diagram the plasmid 5XSRF+Ets-Luc contains 5 tandem repeats of both the Ets- and the SRF-binding sites. The plasmid SRF-Luc is the same but lacks the Ets-binding sites. MIN6 cells were transiently transfected with the indicated constructs and preincubated as described in A and B followed by treatment with 45 mM KCl and 100 ng/ml epidermal growth factor (EGF). Results are shown as means ± SE of 3 independent experiments done in triplicate; *P < 0.04, **P < 0.001.
transcriptional regulation by depolarization in pancreatic β-cells.

Nuclear extracts from MIN6 cells exhibit Elk-1-binding to Ets motifs. The SRE is continuously occupied in vivo by SRF and Ets proteins of the ternary complex subfamily (15, 36, 40). In most cell types examined, formation of this complex is constitutive rather than inducible (15, 48). Previous studies have shown that the sequence encompassing SRE-3 to -5 in the Egr-1 promoter can interact with SRF and Elk-1 proteins at the SRF and Ets-binding domains, respectively (43, 44). Furthermore, Elk-1 in combination with SRF participates in the initiation of Egr-1 gene transcription under other experimental conditions (41). To determine whether Elk-1-binding activity in MIN6 cells is involved in SRE-dependent transcription by depolarization, nuclear extracts from MIN6 cells were incubated with a c-fos SRE. This SRE was chosen as a consensus sequence on the basis of initial gelshift experiments with a probe containing the c-fos SRE and unlabeled Egr-1 SRE-1, -2, -3, -4, and -5. Binding of nuclear protein was competed for by each of the five Egr-1 SREs (data not shown). As observed in other systems, two DNA:protein complexes were observed (Fig. 2A). They included one slower migrating band (band A) and a diffuse faster migrating complex (band B) containing four bands (lane 1). Band A migrated similarly to bands identified by others as the ternary complex composed of the SRE, SRF, and Elk-1 or another TCF (18, 26, 37, 41). The identity of complex B is not known, and the intensity varies with cell type (26). To ensure that some of these complexes contained an Ets family member, competitions with an unlabeled oligonucleotide containing the Drosophila E74A-binding site was performed (34). This site has been shown to bind Elk-1 and compete for TCF binding, thereby inhibiting TCF at the c-fos SRE (21, 28). Bands A and B did not disappear when a nonspecific cold competitor in 5–25, and 50-fold molar excess was used (lanes 2–4). In contrast, complexes A and B were progressively competed for by addition of unlabeled E74A oligonucleotide (lanes 5–7). These results suggested that band A is due to binding of SRF and an Ets family member. The disappearance of some of the components of band B suggested that some of these bands most likely contain homo- or heterodimers of Ets family members. Multiple DNA-protein complexes have also been observed when recombinant Elk-1 was incubated with E74 oligonucleotide (34). To recognize the identity of the proteins present in the SRE complex (bands A and B), nuclear extracts from MIN6 cells were preincubated with anti-SRF and anti-Elk-1 antibody (αSRF and αElk-1, respectively). Addition of αSRF to nuclear extracts resulted in disappearance of band A and appearance of a more slowly migrating band C (Fig. 2B, lane 2). The presence of αElk-1 reduced complex A by 80% as assessed by densitometric analysis (n = 3, Fig. 2B, lane 3), suggesting that Elk-1 is part of the SRE complex. Incubation of the nuclear extracts with a nonspecific antibody (anti-CREB) did not disrupt the protein complex (data not shown). Disruption by αElk-1 of the protein complexes containing Elk-1 has been observed in other systems (1, 8). When nuclear extracts were incubated with an oligonucleotide containing the c-fos SRE with mutated SRF-binding site, no supershift was obtained with αSRF antibody, and a similar decrease in intensity of the bands was observed with the αElk-1 antibody (data not shown). These findings suggest that Elk-1 and the SRF are part of the ternary complex in MIN6 cells.
Depolarization and growth factors induce phosphorylation of Elk-1 in pancreatic β-cells. In other cells, it has been shown that EGF can regulate Elk-1 transcriptional activity by inducing phosphorylation of Ser\(^{383}\) (33). Having shown that depolarization activates Egr-1 transcription via SRE/Ets-dependent interaction and that Elk-1 and SRF are part of the ternary complex in MIN6 cells, we next sought to determine whether Elk-1 is phosphorylated on Ser\(^{383}\) in MIN6 cells after depolarization. Cells were transfected with a plasmid encoding Elk-1 and then subjected to Western blotting with the use of anti-phospho-Ser\(^{383}\) antibodies. Although the effects on phosphorylation of endogenous Elk-1 to depolarization and EGF could be observed, the signals with transfected Elk-1 were more readily evaluated. As shown in Fig. 3A, a rapid Ser\(^{383}\) phosphorylation of Elk-1 by glucose was observed as early as 5 min, which appeared to be diminishing by 30 min. The total amount of Elk-1 protein did not differ with either of the agents tested, as indicated by use of an antibody to nonphosphorylated Elk-1. KCl-induced depolarization resulted in a similar time course of Elk-1 phosphorylation (Fig. 3B).

Activation of phosphorylation of Elk-1 by glucose and KCl at millimolar concentrations could result from osmotic changes. As shown in Fig. 3C, treatment with mannitol (50 mM) failed to result in Elk-1 phosphorylation, as did 3-O-methylglucose (25 mM), a nonmetabolizable glucose analog (data not shown). In contrast, mannose, a metabolizable glucose analog, did activate Elk-1 phosphorylation (data not shown), as did treatment with the depolarizing agent tolbutamide (100 μM). Diazoxide, an islet ATP-activated potassium (K\(_{ATP}\)) channel activator, blocked Elk-1 phosphorylation by 25 mM glucose (data not shown), further substantiating that glucose metabolism followed by inhibition of K\(_{ATP}\) channels and subsequent depolarization are required. Similarly, treatment with the growth factor EGF (100 ng/ml) also resulted in rapid (15 min) Elk-1 phosphorylation to the same extent as tolbutamide.

Depolarization induces Ca\(^{2+}\)-dependent transcriptional activation of Elk-1. To determine whether depolarization-induced Ser\(^{383}\) phosphorylation of Elk-1 is associated with transcriptional activation, a transactivator plasmid Elk1-GAL4 encoding a fusion protein containing Elk-1 transactivation domain (amino acids 307–427) ligated downstream of the sequence encoding the GAL4 DNA-binding and dimerization domain was used. The Elk1-GAL4 plasmid was cotransfected with a reporter gene containing a synthetic promoter with five tandem repeats of the yeast GAL4-binding site upstream of the luciferase gene. As shown in Fig. 4A, KCl-induced depolarization activated Elk-1-dependent...
transcription in a dose-dependent manner. Addition of 25 mM glucose to cells preincubated with 2 mM glucose, a treatment previously shown to result in depolarization-dependent induction of Egr-1 transcription (17), also induced Elk-1-dependent transcriptional activation 2.3-fold ($P < 0.002$, Fig. 4B). Stimulation with EGF (100 ng/ml) resulted in similar transcriptional activation of Elk-1. The results of these experiments indicated that both a nutrient and a growth factor could lead to transcriptional activation of Elk-1.

To evaluate whether the effects of KCl were dependent on extracellular Ca$^{2+}$ influx, the L-type calcium channel inhibitor verapamil was used. As shown in Fig. 4C, depolarization-induced Elk-1 transcriptional activation by KCl (4-fold, $P = 0.01$) was inhibited 88% ($P < 0.001$) by the addition of verapamil at 10 μM and by 100% at 40 μM. No Elk-1 transcriptional activation was observed when verapamil was used alone. These results suggest that depolarization activation of Elk-1 depends exclusively on Ca$^{2+}$ influx from the extracellular compartment.

Elk-1 transcriptional activation is mediated by Ca$^{2+}$-activated Ras/MAPK signaling. Calcium regulation of gene transcription in various cell types involves multiple signaling pathways (15). These include the Ser/Thr kinases PKA, MAPK, and CaMKs. As shown in Fig. 5A, the PKA inhibitor H89 (10 μM) did not inhibit KCl-induced Elk-1-mediated transcriptional activation. Consistent with this result, no response was observed when a plasmid encoding the catalytic unit of PKA was cotransfected. Evidence of the biological activity of this plasmid was previously demonstrated in a CREB-GAL4 transactivation assay (3).

To determine whether MAP kinase activation through Ras signaling is a component of Elk-1 transcriptional activation, plasmids encoding either a wild-type (Ras WT) or a dominant negative form (Ras 15A) were tested. As shown in Fig. 5B, KCl-induced depo-
Fig. 5. Depolarization-induced Elk-1 mediated transcription is not PKA dependent and is mediated by Ca\textsuperscript{2+}-activated Ras signaling. 

A: MIN6 insulinoma cells were transfected with 0.1 ng of Elk-1-GAL4 and 0.25 μg of a reporter plasmid pFR-Luc, as described in EXPERIMENTAL PROCEDURES. After culturing in regular media containing 5 mM glucose and 2% FBS for 24 h, the cells were continued in the same media or stimulated for 6 h with 45 mM KCl either alone or in the presence of H89. The protein kinase A (PKA) inhibitor H89 was added to the culture media to a final concentration of 15 μM 1 h before stimulation. Data are expressed as means ± SE of the fold induction over the luciferase activity at 5 mM glucose. Constitutively active PKA (PKA; 50 ng) was cotransfected where indicated. Data are expressed as means ± SE of the fold induction over the luciferase activity at 5 mM glucose. To control for transfection efficiency, 2 ng of pRL-TK luciferase construct were used in all experiments.

B: effects of Ras in Elk-1-dependent transcription. MIN6 insulinoma cells were transfected as described in A. After culturing in regular media containing 5 mM glucose and 2% FBS for 24 h, the cells were continued in the same media or stimulated for 6 h with 45 mM KCl. Cells were cotransfected with wild-type Ras (Ras WT; 3 μg) or empty vector (vector) where indicated. To control for transfection efficiency, 2 ng of pRL-TK luciferase construct were used. Data are expressed as means ± SE of the fold induction over the luciferase activity at 5 mM glucose.

C: MIN6 cells were transfected with Elk-1 and pFR-Luc with the same concentrations described above. Cotransfection with dominant negative Ras (Ras 15A) (1 or 3 μg) and the corresponding amount of empty vector to maintain a constant DNA concentration was performed. Cells were precultured and induced with 45 mM KCl as described in A. Data are expressed as means ± SE of the percent of KCl induction. Results are representative of 3 independent experiments done in triplicate; *P < 0.04, **P < 0.01, ***P < 0.001.
larization of MIN6 cells transfected with empty vector resulted in a 4.5-fold increase ($P < 0.01$). Transfection with Ras WT resulted in a 6-fold ($P < 0.05$) induction of Elk-1 transcriptional activation, and this response was augmented (14-fold, $P = 0.01$) by KCl-induced depolarization. To assess the role of endogenous Ras in the depolarization activation of Elk-1, cells were cotransfected with a dominant negative Ras (Ras 15A) under

Fig. 6. Depolarization and EGF signaling have common transduction pathways involving extracellular signal-regulated kinase (ERK) activation. A: Elk-1 phosphorylation is mediated by the ERK pathway. MIN6 cells were transfected with an expression plasmid encoding Elk-1 as described in EXPERIMENTAL PROCEDURES. Thirty-six hours later, cells were incubated in KRBH-2% albumin for 1 h followed by stimulation for 15 min with glucose (25 mM) and KCl (45 mM) in the presence or absence of the MEK (PD98059 50 μM) and phosphatidylinositol (PI) 3-kinase (wortmannin 100 μM) inhibitors as indicated in the figure. The MEK and PI 3-kinase inhibitors were added to the media 30 min before stimulation with glucose and KCl. Protein was subjected to Western blotting with a phospho-Elk-1 (p-Elk-1) and Elk-1 antibodies as described. Results are representative of 2 independent experiments done in duplicate. B: Elk-1 transcriptional activation by depolarization and EGF is inhibited by MEK inhibition. Elk-1 transcriptional activation was assessed using the GAL4-Elk-1 luciferase expression system. MIN6 insulinoma cells were transfected with 0.1 ng of Elk-1-GAL4 and 0.25 μg of a reporter plasmid pFR-Luc, as described in EXPERIMENTAL PROCEDURES. After culturing in regular media containing 5 mM glucose and 2% FBS for 24 h, the cells were continued in the same media or stimulated for 6 h with KCl (45 mM) and EGF (100 ng/ml) in the presence or absence of the MEK (PD98059, 50 μM) and p38 (SB203580, 5 μM) inhibitors. The MEK and p38 inhibitors were added to the media 1 h before stimulation with KCl or EGF. To control for transfection efficiency, 2 ng of pRL-TK luciferase construct were used. Data are expressed as means ± SE of the fold induction over the luciferase activity at 5 mM glucose. Results are representative of 3 independent experiments done in triplicate; *$P < 0.01$, **$P < 0.001$. C: measurement of Erk activity after glucose and KCl treatment. Cells were preincubated for 1 h in KRBH-2% albumin followed by stimulation with 25 mM glucose and 45 mM KCl for the indicated time points. Cell lysates were immunoprecipitated with anti-phospho-ERK-1 and -2 specific antibodies. ERK activity was measured by incubation of the immunoprecipitate with recombinant Elk-1, followed by immunoblotting using an anti Ser$^{383}$ phospho-Elk-1 antibody as described in EXPERIMENTAL PROCEDURES. Results are representative of 2 independent experiments performed in duplicate.
conditions similar to those that others have shown to be effective in inhibiting Ras signaling (6) (Fig. 5C). A partial dose-dependent inhibition was observed to 86 (P = 0.006) and 58% (P < 0.001) of control. Failure to observe complete inhibition in depolarization induction of Elk-1 transcriptional activation by inhibition of Ras signaling suggested that perhaps other Ca2+-activated pathways are involved (see below). This idea was also supported by the augmentation by KCl treatment of Elk-1 transcriptional activity in Ras WT-transfected cells (Fig. 5B).

Previous studies have shown that glucose and Ca2+ influx induces MAPK activation (16, 23, 32). To determine whether Elk-1 phosphorylation induced by glucose and KCl is mediated by ERK-1/2 activation, the specific MEK inhibitor PD98059 was used. Both glucose- and KCl-induced depolarization resulted in Elk-1 phosphorylation on Ser383 that was abolished by PD98059 (Fig. 6A), suggesting that this pathway is a major mediator in this response. Although PI 3-kinase activation has been implicated in β-cell proliferation induced by IGF and glucose (19), the presence of the PI 3-kinase inhibitor wortmannin did not result in altered glucose activation of Elk-1 Ser383 phosphorylation (Fig. 6A). By use of the Elk-1 transactivation assays, both KCl and EGF activation were also shown to be blocked by treatment with the specific MEK inhibitor PD98059, as shown in Fig. 6B.

Although ERK-1 and -2 appear to be the major Elk-1 kinases regulated by growth factors in noninsulinoma cells, c-Jun-NH2-terminal kinase (JNK) and p38 kinases are also capable of activating Elk-1 in response to cellular stresses and cytokines (5, 45–47). The related MAPK family members JNK/stress-activated protein kinases are insensitive to glucose and Ca2+ influx in insulinoma cells (23). In contrast, the p38 MAPK has been shown to be activated by glucose and to be involved in IUF1-dependent gene transcription in MIN6 insulinoma cells (23, 27). To determine the possible contribution of p38 kinase in depolarization activation of Elk-1-dependent transcription, MIN6 cells transfected with the Gal-Elk-1/Gal-luciferase expression-reporter system were evaluated in the presence of the specific p38 inhibitor SB203580. The p38 inhibitor

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**Fig. 7.** Elk-1 transcription activation by CaMKIV is MAPK dependent. A: effects of activation of CaMKIV and -II pathways in Elk-1-dependent transcription. Elk-1 transcriptional activation was assessed using the GAL4-Elk-1 luciferase expression system. MIN6 insulinoma cells were transfected with 0.1 ng of Elk-1-GAL4 and 0.25 μg of a reporter plasmid pFR-Luc, as described in EXPERIMENTAL PROCEDURES. After culturing in regular media containing 5 mM glucose and 2% FBS for 24 h, the cells were continued in the same media (5 mM glucose) or stimulated for 6 h (45 mM KCl). Cells were cotransfected with wild-type 1 μg of constitutively active Ca2+/calmodulin kinase (CaMKIV) or 1 μg of constitutively active CaMKII (CaMKIIBC) as indicated. A corresponding amount of empty vector was used to maintain a constant DNA concentration. To control for transfection efficiency, 2 ng of pRL-TK luciferase construct were used. Data are expressed as means + SE of the fold induction over the luciferase activity at 5 mM glucose. B: CaMKIV induction of Elk-1-dependent transcription is MAPK dependent. MIN6 insulinoma cells were transfected with the GAL4-Elk-1 activation system as described in A and B. Cotransfection with 1 μg of CaMKIV or 50 ng of MEKK (inset) alone or with 50 μg of a plasmid encoding for the MAP kinase phosphatase 1 (MKP1). DNA concentration was maintained constant by using the corresponding empty plasmids. To control for transfection efficiency, 2 ng of pRL-TK luciferase construct were used in all experiments. Data are expressed as means + SE of the fold induction over the luciferase activity at 5 mM glucose. Values are representative of 3 independent experiments done in triplicate; *P < 0.01.
failed to reduce depolarization-induced transcriptional activation of Elk-1 compared with cells treated with the inhibitor alone (Fig. 6B). To confirm that the findings obtained by Western and transcriptional activation assays correlated with activation of the MAPK, we measured ERK-1/2 enzymatic activity after treatment with either glucose or KCl. Both glucose and KCl treatment resulted in increased ERK activity as early as 5 min, whereas that induced by KCl appeared to be of greater magnitude, perhaps due to the more marked effect on Ca\(^{2+}\) influx (Fig. 6C).

**CaMKIV activates Elk-1-dependent transcription.** Previous evidence obtained with Ca\(^{2+}/\mathrm{CaM}\) inhibitors suggested that activation of Egr-1 transcription in insulinoma cells by depolarization involved this pathway (3). To determine whether activation of CaMKIV and CaMKII, in addition to the Ras/MAPK pathways, modulate Elk-1-dependent transcription, plasmids encoding constitutively active forms of these kinases were assessed in the GAL4-Elk-1 transcriptional activation assay. Transfection with CaMKIVCT or CaMKIIBC, respectively, activated Elk-1-dependent transcription 5- (P = 0.01) and 2.3-fold (P = 0.002), respectively (Fig. 7A). Neither dominant negative forms of CaMKIV (CaMKIVKT) nor CaMKII (CaMKIIBI) inhibited the depolarization induction of Elk-1-dependent transcription (data not shown). These results may be explained by the alternative activation through the Ras pathway. That both pathways do contribute to depolarization activation of Elk-1 was suggested by an additional 25% inhibition of Elk-1 activation with cotransfection of a dominant negative CaMKIV together with the dominant negative Ras (data not shown).

In neuronal cells (NG108), Ca\(^{2+}\) influx activates Elk-1 via two pathways that converge on Ras/Raf/MEK. One involves Ca\(^{2+}\) activation of a soluble tyrosine kinase receptor, the other Ca\(^{2+}\) activation of CaMKIV (13). To determine whether the ERK pathway is involved in the activation of Elk-1 by CaMKIV, cotransfection with a plasmid encoding a specific inhibitor of the ERK pathway, MKP-1, was performed. In Fig. 7B is shown the complete inhibition of CaMKIV induction of ELK-1 transcriptional activation by MKP-1. The effectiveness of the MKP was demonstrated by >95% inhibition of Elk-1 transcription when cotransfection of MKP-1 and a plasmid encoding MEKK was performed (see Fig. 7B, inset). The results of the experiments described above suggest that CaMKIV, as well as Ras, also activates Elk-1-dependent transcription by activation of the MAPK pathway.

**DISCUSSION**

A common cellular response to a variety of growth stimuli is the activation of various kinase-dependent signaling pathways. Several of these kinase-mediated pathways have been shown to be triggered by glucose treatment of pancreatic β-cells. Although it is recognized that hyperglycemia serves as a growth factor for these cells, the proximal mechanisms whereby glucose-induced depolarization and Ca\(^{2+}\) influx alter gene expression are still undetermined. To appreciate the mechanisms concerned in Ca\(^{2+}\) regulation of islet β-cell transcription, we have focused on the identification of signal transduction pathways by which Ca\(^{2+}\) leads to the phosphorylation and activation of transcription factors regulating the Egr-1 promoter. In the
current studies, we demonstrated 1) that the Ets elements are important components in depolarization-activated SRE-dependent Egr-1 transcription, 2) that Elk-1 is one of the TCFs that bind the SRE in pancreatic β-cells, 3) that Elk-1 is phosphorylated and transcriptionally activated by glucose and other depolarizing agents, as well as by growth factors, and 4) that the MEK/ERK pathway is involved in Elk-1-dependent transcription activated by both glucose and growth factors. These studies also confirm the tissue specificity of Ca$^{2+}$-dependent TCF-mediated transcription, since similar findings were described in cortical neurons and the pituitary cell line AtT20, but not in hippocampal neurons or PC12 cells (2). Most important, the results of these studies are the first to show the convergence of glucose-induced depolarization and growth factor treatment on activation of a transcription factor in insulinoma cells.

It was noted that KCl-induced depolarization increased both phosphorylation and transactivation of Elk-1 to a greater extent than glucose treatment. The differences in the responses between glucose and depolarization can be explained by differences in the magnitude of the stimulus, which we have previously shown is extracellular Ca$^{2+}$. This conclusion is based on the observation that inhibition of both glucose and KCl induction of Egr-1 transcription was blocked by the hyperpolarizing agent diazoxide and by Ca$^{2+}$ channel blockers (3). Because it was previously shown that KCl-induced depolarization elicited a higher magnitude of Ca$^{2+}$ influx, we decided to use this stimulus to study the signaling pathways induced by depolarization.

Efforts to define the proximal pathways whereby Ca$^{2+}$ influx triggers the MEK/ERK pathway involved transfections with Ras and CaMK plasmids. Although expression of wild-type Ras resulted in robust Elk-1 transcriptional activation, expression of a dominant negative form of Ras, under conditions where others have observed marked inhibition (6), resulted in only partial inhibition. These results suggested that other pathways besides Ca$^{2+}$ activation of Ras could be involved. In the present study, we demonstrated that activation of the CaMKIV pathway resulted in Elk-1-dependent transcriptional activation. This activation was ERK dependent as suggested by the complete inhibition of CaMKIV transactivation of Elk-1 by the ERK-1-specific MAP kinase phosphatase (Fig. 7B). Similar activation of the ERK pathway by CaMKIV has been shown in NG108 neuronal cells (13). Although the present study showed that activation of the CaMKIV pathway resulted in Elk-1-dependent transcriptional activation, the lack of inhibition by a dominant negative form of CaMKIV raised the concern about the role of endogenous CaMKIV. An additional inhibition was observed when a combination of dominant negative forms of Ras and CaMKIV was used, however (data not shown). The results of these experiments suggested that the CaMKIV pathway contributes to, but is not essential for, depolarization induction of Elk-1 in insulinoma cells.

EGF has been shown to modulate islet growth in animal models, and overexpression of EGF in β-cells in transgenic mice results in pancreatic β-cell hyperplasia (24). Recently, EGF receptor-deficient mice were shown to have generalized defects in islet β-cell proliferation (29). EGF activation of Elk-1 transcription has been demonstrated in several cell types. In the present studies, we showed that EGF treatment of MIN6 insulinoma cells results in Elk-1 activation. As demonstrated in other systems, this activation occurs via a Ras/MAPK pathway, as this effect was inhibited by PD98059. The results of the present studies also indicated a convergence of glucose/depolarization/Ca$^{2+}$ activation and EGF-mediated signaling pathways through activation of Ras/MAPK/ERK-1/2. Thus both mitogenic stimuli result in transcriptional activation of Elk-1 via similar mechanisms. In this regard, in preliminary experiments we have demonstrated that, in insulinoma cells, inhibition of the MEK MAPK pathway by PD98059 decreases thymidine incorporation induced by both EGF and glucose (data not shown). The present studies thus provide an in vitro experimental model to begin to assess the molecular mechanisms involved in EGF responses in transgenic animals.

On the basis of the results of previous (3) and the present studies, a schematic representation of the signal transduction pathways involved in depolarization/Ca$^{2+}$ regulation of SRE-dependent transcription in pancreatic β-cells is suggested in Fig. 8. Using the Egr-1 gene as a model, we have shown that the Egr-1 promoter contains five SREs and that the transcriptional response to depolarization is SRE dependent. In response to increased glucose metabolism, depolarization and Ca$^{2+}$ influx result in CaMKIV activation of SRF (3). The present studies now demonstrate Ca$^{2+}$/CaM/CaMKIV activation of another transcription factor, Elk-1, involved in SRE-mediated transcription. In contrast to the activation of SRF by CaMKIV, Elk-1-dependent transcription requires activation of the Ras/MEK pathway. EGF activation of Elk-1-dependent transcription also requires the Ras/MEK pathway. Thus the results of the present experiments show that both glucose/depolarization and EGF treatment converge on a common pathway leading to Elk-1 activation. How this pathway contributes to the proliferation of β-cells to prolonged hyperglycemia and insulin resistance can now be tested in different experimental models.

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